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ELECTROGENIC PROTON EJECTION COUPLED TO ELECTRON TRANSPORT THROUGH THE ENERGY-CONSERVING SITE 2 AND K^+/H^+ EXCHANGE IN YEAST MITOCHONDRIA

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The proton ejection coupled to electron flow from succinate and/or endogenous substrate(s) to cytochrome *c* using the impermeable electron acceptor ferricyanide is studied in tightly coupled mitochondria isolated from two strains of the yeast *Saccharomyces cerevisiae*. (1) The observed H^+ ejection/$2e^-$ ratio approaches an average value of 3 when K^+ (in the presence of valinomycin) is used as charge-compensating cation. (2) In the presence of the proton-conducting agent carbonyl cyanide *m*-chlorophenylhydrazone, an H^+ ejection/$2e^-$ ratio of 2 is observed. (3) The low stoichiometry of $3H^+$ ejected (instead of 4) per $2e^-$ and the high rate of H^+ back-decay (0.1615 lnΔ-(ngatom)H^+/s and a half-time of 4.6 s for 10 mg protein) into the mitochondrial matrix are related to the presence of an electroneutral K^+/H^+ antiporter which is demonstrated by passive swelling experiments in isotonic potassium acetate medium.

Introduction

In yeast mitochondria, the electrogenic H^+ ejection coupled to electron flow from different substrates to oxygen has been measured with the oxygen-pulse technique described by Mitchell and Moyle [1,2]. Values close to $2H^+$ ejected per $2e^-$ and per energy-conserving site were obtained [3–8]. However, the H^+ ejection coupled to one separate span of the respiratory chain of yeast mitochondria has not yet been studied. We have investigated the H^+ ejection coupled to electron flow from succinate and/or endogenous substrate(s) to cytochrome *c* using ferricyanide as artificial electron acceptor in *Saccharomyces cerevisiae* mitochondria. We obtain an average value of $3H^+$ ejected per $2e^-$. This stoichiometric ratio is most probably underestimated because of the operation of a K^+/H^+ electroneutral exchange. The existence of an active K^+/H^+ antiporter is demonstrated by uncoupler-insensitive passive swelling in isotonic potassium acetate medium.

Materials and Methods

Yeast strain. The D-261 and KL14-4A strains of the yeast *S. cerevisiae* were received from Dr. J. Mattoon (Denver University, Colorado Springs, U.S.A.) and Dr. P. Slonimski (Centre de Génétique Moléculaire, Gif-sur-Yvette, France), respectively.

Growth conditions. Five Erlenmeyers flasks of 5 l each containing 1 l of 2% (w/v) yeast extract (KAT, Ohly, Hamburg, F.R.G.), 0.2% (w/v) KH_2PO_4, 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) glucose, 3% (v/v) ethanol, 0.4 g penicillin and 70 mg streptomycin were inoculated at $2 \cdot 10^6$ cells/ml from a preculture and agitated at 30°C for 18–20 h until the culture reached a density of $135–150 \cdot 10^6$ cells/ml.

Preparation of mitochondria. The washed cells were incubated for 1 h at 33°C in 600 ml of 0.1 M Tris-HCl, 0.1 M mercaptoethanol (pH 8.0). These

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetracetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

cells washed twice with distilled water and incubated for 1–2 h at 33°C in 800 ml of 1 M sorbitol, 0.5 mM EDTA, 10 mM imidazole hydrochloride (pH 6.4) and 5 ml glusulase (Endo Laboratory, Inc., NY) until protoplasts were obtained. The protoplasts were washed three times by centrifugation at 1900 × *g* during 5 min in 1.3 M sorbitol, 0.5% (w/v) bovine serum albumin, 0.5 mM EDTA, 10 mM imidazole hydrochloride (pH 6.4), suspended in 200 ml of 0.6 M mannitol, 0.1% (w/v) bovine serum albumin, 10 mM imidazole hydrochloride (pH 6.4), and homogenized manually in appropriated batches with five strokes in a Dounce homogenizer. The homogenate was centrifuged at 1900 × *g* for 5 min. The supernatant was centrifuged at 17 000 × *g* for 10 min. The pellet was cleaned of its fluffy layer and of the lipid material adhering to the tube walls. The mitochondrial fraction was washed in 0.6 M mannitol, 0.1% (w/v) bovine serum albumin, 10 mM imidazole hydrochloride, (pH 6.4) and centrifuged again at 17 000 × *g* for 10 min. The pellet was resuspended in a small volume of the same medium. The final protein concentration was 60–80 mg protein/ml, and the material was used immediately.

Instrumental measurements. Oxygen consumption was measured at 25°C with a Clark-type electrode (Rank Brothers, Cambridge, U.K.) in a closed thermostatically controlled chamber of 3 ml with magnetic stirring. H^+ movements were measured in a thermostatically controlled chamber with a high-sensitive small-diameter combination pH-glass electrode (A.H. Thomas Co. model 4094-L25). The electrode outputs were amplified through a Beckman Expandomatic SS-2 pH-meter and fed into a dual-channel Tekman Electronics Ltd. recorded adjusted to adequate chart speed. Known amounts of standard solutions of HCl and KOH were added to calibrate the pH-electrode response in all the experiments. Mitochondrial swelling was monitored by the decrease of absorbance at 750 nm using a Perkin-Elmer 550 spectrophotometer.

Proteins were measured as described by Murphy and Kies [9]. All the reagents were added in small volume of stock solutions adjusted to the same pH as that of the medium except for CCCP, oligomycin, valinomycin and antimycin A, which were prepared in methanol. Bovine serum albumin fraction V (Sigma, St Louis, U.S.A.) was washed several times in absolute ethanol and dried under vacuum before use.

Results

As indicated in the legends to the figures, the respiratory control ratios of the mitochondria used in this study were very high. However, when K^+ at a high concentration (100 mM) was added to the respiratory medium, the rate of ADP-stimulated respiration was markedly decreased. This observation prompted us to examine further the relationships between K^+ movements and the proton-motive force generated by respiration. In these well coupled mitochondria, the addition of Ca^{2+} in the presence of P_i and Mg^{2+} did not stimulate the respiratory rate, confirming that yeast mitochondria do not efficiently transport Ca^{2+} [10,11]. Therefore, Ca^{2+} could not be used as charge-compensating cation in the H^+-ejection experiments.

Electrogenic H^+ ejection coupled to electron transport through the energy-conserving site 2 and rate of H^+ back-decay

Fig. 1 shows a typical experiment of H^+ ejection coupled to electron flow from succinate to cytochrome *c*. The mitochondria were preincubated in the presence of KCN to inhibit electron flow through the cytochrome oxidase. Mersalyl was added to prevent the uptake of phosphate via the P_i^-/OH^- antiporter. Valinomycin was added to make the inner mitochondrial membrane permeable to K^+. After a few minutes of preincubation, a small pulse of a known amount of ferricyanide was added and H^+ ejection recorded. The initial H^+ ejection observed during a few seconds was followed by a very rapid partial H^+ reentry into the mitochondrial matrix. In a series of nine experiments, the rate of H^+ back-decay into the mitochondrial matrix was calculated from a plot of $\ln\Delta[H^+]$ vs. time. The average value of $\ln\Delta(\text{ngatom})H^+/s$ was 0.1615 ± 0.049 and the average half-time ($t_{1/2}$) of H^+ reentry into the mitochondrial matrix was 4.66 ± 1.50 s for 10 mg protein.

Before the addition of ferricyanide, a slow but constant release of H^+ into the external medium was observed. This H^+ release is not linked to electron flow through the cytochrome oxidase because of the presence of CN^-, nor to electron flow through a CN^--insensitive respiration, since it was also observed under N_2. Under anaerobic conditions, a similar rate and extent of H^+ ejection upon addition of ferricyanide were obtained (not shown).

Only a small extrapolation was required to calculate the total observed H^+ ejection (164.3 ngatom H^+ for 100 nmol of ferricyanide) in the experiment of Fig. 1 which yielded an H^+ ejection/$2e^-$ ratio of 3.28. In the presence of CCCP, the rate of H^+ ejection was smaller and the total extent of H^+ ejection decreased to 100 ngatom H^+. This value corresponds to $2H^+$ translocated per $2e^-$ transported vectorially from the inner side (succinate dehydrogenase) to the outer side (cytochrome *c*) of the inner mitochondrial membrane. Antimycin A inhibits up to 95% of the electron flow as well as the coupled H^+ ejection in the presence of CCCP. This confirms that ferricyanide takes electrons from the outer side of the inner mitochondrial membrane and does not react directly with the dehydrogenase [13] located at the inner side of the inner mitochondrial membrane. After the H^+ back-decay is completed, the external pH does not generally reach exactly the same value as in the control experiment carried out in the presence of CCCP. This might indicate a slightly more acid pH in the matrix than in the external medium before the addition of ferricyanide. The H^+ ejection upon addition of three consecutive pulses of ferricyanide yields a similar H^+ ejection/$2e^-$ ratio (not shown).

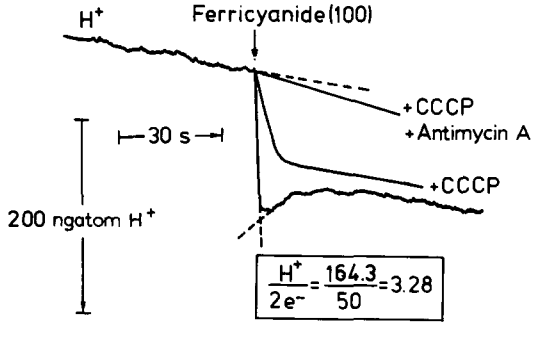

Fig. 1. H^+ ejection coupled to electron flow in the succinate-ferricyanide span of yeast mitochondria. The reaction medium (3.0 ml at 25°C) contained: 0.52 M mannitol, 2 mM imidazole (pH 6.2), 0.1% (w/v) bovine serum albumin, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 3.33 mM NaCN, 0.42 mM succinate (sodium salt), 0.32 mM mersalyl, 2 μg valinomycin and 10 mg mitochondrial protein (strain D-261). The reaction was started with a pulse of 100 nmol potassium ferricyanide. Where indicated, 6.6 μM CCCP or 1.66 μM antimycin A was added. The respiratory control ratio was 3.7 with succinate as substrate.

Table I presents the average ± standard deviation of the H^+ ejection/$2e^-$ ratio as observed from a large number of mitochondrial preparations of the yeast *S. cerevisiae* strains D-261 and KL14-4A under a variety of experimental conditions. In the absence of added substrate, the addition of ferricyanide causes a significant burst of H^+ ejection, indicating that electrons were donated from endogenous substrate(s). Since yeast mitochondria isolated from cultures at the end of the exponential phase of growth lack the energy-conserving site 1 [12,13], the H^+ ejection coupled to the oxidation of endogenous substrate(s) is likely to take place via the segment of the respiratory chain corresponding to the energy-conserving site 2, as in the case of succinate.

Omission of mersalyl or *N*-ethylmaleimide (inhibitors of the P_i^-/OH^- antiport carrier) results only in a slight decrease of the observed H^+ ejection/$2e^-$ ratio. If valinomycin was omitted, the H^+ ejection/$2e^-$ ratio became close to 2, which is the value obtained in the control experiments carried out in the presence of CCCP.

A K^+/H^+ electroneutral exchange reaction in yeast mitochondria

The H^+ ejection/$2e^-$ ratio observed in our experiments varies from 2.2 to 3.5. This variation depends on the mitochondrial preparation, but is not related to the variation of the respiratory control ratio. These results contrast with those obtained in mammalian mitochondria [14–18] and in the cytochrome bc_1 complex incorporated into liposomes [19] where an H^+ ejection/$2e^-$ ratio of 4.0 is currently observed for the same respiratory span. A K^+/H^+ electroneutral exchange could account for partial recycling of H^+ and K^+ and would decrease the H^+ ejection/$2e^-$ ratio. This possibility was tested by measurements of passive swelling in different isotonic media supplemented with antimycin A and oligomycin. Fig. 2A shows that, as expected [20], fast swelling is induced in isotonic ammonium acetate medium, while in calcium acetate little swelling is observed. Very fast and extensive swelling takes place in isotonic potassium acetate medium. Similar swelling, although proceeding at a slower rate, is observed in isotonic sodium acetate medium. The swelling in potassium acetate is not further stimulated by addition of the H^+-conducting agent CCCP (Fig. 2B), indicating that the K^+ influx

TABLE I

AVERAGE H^+ EJECTION/$2e^-$ RATIO IN THE ENERGY-CONSERVING SITE 2 IN DIFFERENT PREPARATIONS OF YEAST MITOCHONDRIA AND UNDER DIFFERENT ASSAY CONDITIONS

The data given here are the mean ± S.D. of the number of experiments indicated between brackets. The experiments were carried out as indicated in Fig. 1. However, some assays were carried out in N_2-saturated medium. In other experiments, the concentrations of K^+, valinomycin, succinate (sodium or potassium salt), cyanide, mersalyl or *N*-ethylmaleimide and mitochondrial protein were modified in a wide range. Also, experiments were performed in the absence or presence of $MgCl_2$, EGTA and oligomycin. The ferricyanide addition was 5–10 nmol/mg protein and the pH of the medium ranged from 6.1 to 7.0. None of these modified conditions changed significantly the H^+ ejection/$2e^-$ ratio. Therefore, these assays were pooled to calculate the average value.

Strain	*N*-Ethylmaleimide or mersalyl	Other conditions	H^+ ejection/$2e^-$ ratio
D-261	+	–	2.71 ± 0.40 (57)
	–	–	2.46 ± 0.50 (2)
	+	Endogenous substrate(s)	2.40 ± 0.17 (2)
	+	No valinomycin	2.03 ± 0.00 (2)
	+	+ CCCP	1.92 ± 0.12 (3)
KL14-4A	+	–	2.38 ± 0.14 (10)
	–	–	2.32 ± 0.11 (2)
	–	Endogenous substrate(s)	2.44 ± 0.09 (6)
	–	+ CCCP	2.00 ± 0.04 (3)

does not take place via a uniport. However, addition of valinomycin alone stimulates the rate of swelling and when a K^+/H^+ exchange is artificially induced by the combined addition of CCCP and valinomycin, a very fast swelling is observed.

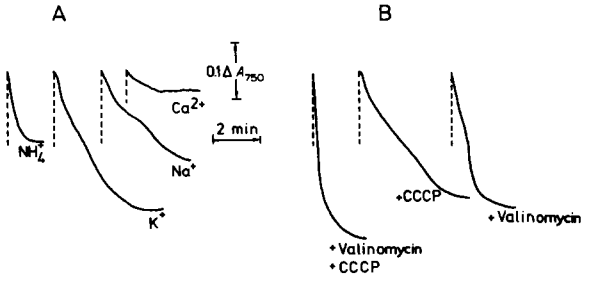

Fig. 2. Passive swelling of yeast mitochondria in different isotonic acetate media. The reaction was started by addition at room temperature of 1 mg mitochondrial protein (strain D-261) in 20 μl of 0.6 M mannitol, 0.1% (w/v) bovine serum albumin, 10 mM imidazole hydrochloride (pH 6.4) to 3 ml of the reaction mixture containing 600 mosM of the indicated acetate salt, 10 mM Hepes (pH 7.0), 1.66 μM antimycin A and 5 μg oligomycin. Where indicated, 3.3 μM CCCP and 5 μg of valinomycin were also added. Experiments where mitochondria were pretreated with antimycin A and oligomycin yielded similar results. The respiratory control ratio ranged from 3.3 to 3.7 using succinate.

Discussion

The rapid burst of H^+ ejection observed upon addition of ferricyanide comprises two components that can be distinguished by their sensitivity to uncouplers. The H^+ ejection insensitive to CCCP gives a stoichiometric H^+ ejection/$2e^-$ ratio of 2.0. This results is expected, since the vectorial movement of two electrons from the inner to the outer side of the inner mitochondrial membrane is responsible for the charge-compensating movement of $2H^+$ extruded into the external medium.

In our experimental system, the net electronic H^+ ejection (above the CCCP control) is identified by its reentry into the mitochondrial matrix (Fig. 1) due to the developing ΔpH (alkaline inside) that takes place during H^+ ejection in the presence of the charge-compensating cation K^+ (+valinomycin). The most evident difference between our results and those reported in mammalian systems in the same respiratory span [14–19] is the lower value of the H^+ ejection/$2e^-$ ratio measured in yeast mitochondria (3 as against 4). Furthermore, the rate of H^+ reentry into the mitochondrial matrix appears to be much faster in yeast than in rat liver mitochondria, as shown by a comparison of Fig. 1 in this paper with similar figures in

Refs. 15–17. Both observations could be easily explained if in yeast a partial H^+ recycling took place during electron flow under our experimental conditions. The fast and extensive CCCP-insensitive passive swelling observed in isotonic potassium acetate medium suggests the presence in the yeast mitochondrial inner membrane of a very rapid K^+/H^+ electroneutral exchange.

In the experiments on H^+ ejection previously described in yeast mitochondria, using the oxygen pulse technique [3–8] the H^+ ejection/O ratio was probably also underestimated because of the unnoticed K^+/H^+ exchange. The rate of H^+ back-decay into the yeast mitochondrial matrix observed under our experimental conditions is, however, much higher than the values reported previously with the oxygen pulse technique [7] in spite of the higher respiratory control ratio of our mitochondrial preparations. This discrepancy could indicate a different activity of the K^+/H^+ exchange under different experimental conditions and/or when using different yeast strains.

Finally, we would like to comment on some implications of the existence in yeast mitochondria of a K^+/H^+ electroneutral antiporter. Evidence for such a carrier has also been reported for mammalian [21,22, 24–29] as well as plant mitochondria [30]. The operation of such a carrier preserved the difference in electrical potential ($\Delta\psi$) generated across the inner mitochondrial membrane by the electrogenic H^+ ejection coupled to electron flow. Therefore, the K^+/H^+ carrier has no uncoupling function and preserves oxidative phosphorylation. The electroneutral exchange of K^+ for H^+ might be a physiological pathway for mitochondrial K^+ efflux while for K^+ influx an electrophoretic pathway has been suggested [23]. The simultaneous function of both carriers will result in the uncoupling of oxidative phosphorylation if they proceed at sufficient rates. Therefore, some sort of control of the rate of these K^+ movements must exist, as already suggested for mammalian mitochondria [25–29,31].

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