Does Any Yeast Mitochondrial Carrier Have a Native Uncoupling Protein Function?

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In this study, we explore the hypothesis that some member of the mitochondrial carrier family has specific uncoupling activity that is responsible for the basal proton conductance of mitochondria. Twenty-seven of the 35 yeast mitochondrial carrier genes were independently disrupted in *Saccharomyces cerevisiae*. Six knockout strains did not grow on nonfermentable carbon sources such as lactate. Mitochondria were isolated from the remaining 21 strains, and their proton conductances were measured. None of the 21 carriers contributed significantly to the basal proton leak of yeast mitochondria. A possible exception was the succinate/fumarate carrier encoded by the *Xc2* gene, but deletion of this gene also affected yeast growth and respiratory chain activity, suggesting a more general alteration in mitochondrial function. If a specific protein is responsible for the basal proton conductance of yeast mitochondria, its identity remains unknown.

INTRODUCTION

Not all the energy released during mitochondrial substrate oxidation is coupled to ATP synthesis. Some is dissipated as heat when protons pumped out of the matrix during electron transport return through proton conductance pathways in the inner membrane (Brand *et al.*, 1999; Stuart *et al.*, 2001a). Mammalian brown adipose tissue mitochondria contain a specialized, cold-inducible uncoupling protein, UCP1, which catalyzes proton leak and leads to proton cycling and regulated thermogenesis. UCP1 is restricted to brown adipose tissue, but other, basal, proton conductance pathways are present in all mitochondria. The proton cycling caused by these basal pathways causes a significant proportion of cellular metabolic rate: 22% in stimulated rat hepatocytes and 34% in contracting rat skeletal muscle. Proton cycling may be responsible for 15–20% of the basal metabolic rate of the rat (Rolfe *et al.*, 1999). It appears to be equally prominent in hepatocytes of all species that have been investigated, regardless of body mass (Porter and Brand, 1995) or phylogenetic position (Brand *et al.*, 2001), suggesting that it accounts for an important part of standard metabolic rate in all animals. Proton cycling may also be significant in bacteria (Albers *et al.*, 2001) and yeast. Yeast incubated with glucose makes less than half of the expected ATP for each oxygen consumed, perhaps because of high proton leak rates of yeast mitochondria in vivo under these conditions (Sheldon *et al.*, 1996).

The mechanism of basal proton leak is unclear. The proton leak current depends nonlinearly on its driving force (membrane potential), increasing greatly at higher potentials (Nicholls, 1974). This nonohmic relationship is expected for noncatalyzed ion diffusion across a phospholipid bilayer (Garlid *et al.*, 1989) and has been found experimentally in artificial phospholipid membranes (see Deamer and Nichols, 1989). The fatty acyl composition of the mitochondrial inner membrane phospholipids correlates with proton conductance (Brookes *et al.*, 1998; Fontaine *et al.*, 1996; Porter *et al.*, 1996). These observations suggest that mitochondrial proton conductance is merely a physical property of the phospholipid bilayer.

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However, the mitochondrial inner membrane is much more permeable to protons than the phospholipid bilayer (see Stuart *et al.*, 2001a). The proton conductance of liposomes made from inner membrane phospholipids from different species was only 2.5–25% of the proton conductance of the mitochondria they were derived from (see Brand *et al.*, 2001). Unlike mitochondrial proton conductance, liposome conductance did not correlate with phospholipid fatty acyl composition (Brookes *et al.*, 1997, 1998). These observations suggest that simple diffusion of protons through the bulk phospholipid of the inner membrane does not fully explain mitochondrial proton conductance. Explanations of the much greater proton permeability of the native inner membrane include nonspecific effects of membrane proteins on the physical properties of the membrane, or the existence of specific protein(s), analogous to UCP1, that catalyze proton conductance.

The yeast *Saccharomyces cerevisiae* provides a good system to test the second possibility, because the genome is sequenced and relatively well-characterized. It encodes 35 putative members of the mitochondrial carrier family (El Moualij *et al.*, 1997; Nelson *et al.*, 1998), which (in mammals) includes the uncoupling protein UCP1. Yeast lacks close sequence homologues of UCP1, yet yeast mitochondria have normal basal proton conductance (Brand *et al.*, 1999). In this paper we examine the hypothesis that some other member of the mitochondrial substrate carrier family is a specific uncoupling protein that is responsible for the basal proton conductance of yeast mitochondria.

The mitochondrial substrate carriers constitute a family of proteins with related primary structures. They have three tandem-repeated sequences of about 100 amino acids, each consisting of two hydrophobic transmembrane α-helices joined by a long segment on the matrix side containing the sequence motif P.(x).D/E.($2x$).K/R.(x).R/K.(10-$30x$).E/D.G.($4x$).F/Y/W.K/R.G (Walker and Runswick, 1993). Table I lists the 35 yeast genes. Currently, 16 of the 35 proteins have known transport functions. Table I shows the suspected transport substrate of three others: VIIIc1—coenzyme A; XIVc1 and XVIc3—aspartate/glutamate (by homology with human aralar2/citrin protein).

We have looked for a putative catalyst of basal proton conductance within the mitochondrial carrier family for several reasons. (1) Members of the family are present in all mitochondria. (2) UCP1, the only well-characterized endogenous protein uncoupler, is a family member. (3) The UCP1 homologues, UCP2 and UCP3, catalyze superoxide-dependent inducible proton leak in mammalian mitochondria (Echtay *et al.*, 2002). In addition, skeletal muscle mitochondria from mice lacking UCP3 are reported to have decreased basal proton conductance

(Gong *et al.*, 2000; Vidal-Puig *et al.*, 2000), although we find they do not (Cadenas *et al.*, 2002). Identifiable homologues of the mammalian UCPs are absent from the yeast genome (Brand *et al.*, 1999). (4) Several other family members, such as the adenine nucleotide, aspartate/glutamate, dicarboxylate, and phosphate transporters, catalyze AMP-dependent (Cadenas *et al.*, 2000) or fatty acid-dependent proton leak (see Skulachev, 1998), although the physiological importance is unclear. (5) The other obvious candidates, the respiratory chain complexes and the ATP synthase, do not contribute significantly through turnover-dependent pathways (Brown and Brand, 1991).

In this paper we report the effect of independent disruption of mitochondrial carrier genes in *S. cerevisiae* on mitochondrial proton leak. We conclude that 20 of the 21 carriers tested make no significant contribution to the basal proton conductance of yeast mitochondria. The succinate/fumarate carrier might make a small contribution, but its phenotype is ambiguous.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Deletion of yeast nuclear genes encoding mitochondrial carrier proteins was accomplished by homologous recombination of the auxotrophic marker *HIS3* (*URA3* for *OAC1*) at the corresponding loci of *S. cerevisiae* strain YPH499 (wild type: *MATa*, *ade2*-101, *his3*-Δ200, *leu2*-Δ1, *ura3*-52, *trp1*-Δ63, *lys2*-801) and verified by polymerase chain reaction. The *XIIc1* deletant was made by Palmieri *et al.* (1999c). Yeast cells were precultured aerobically at 30°C on synthetic complete medium (Sherman, 1991) supplemented with 0.12% ammonium sulfate, 0.1% potassium phosphate, and 2% (w/v) DL-lactic acid as the only carbon source. Histidine and uracil were omitted where the genotype permitted. Precultures were diluted 50-fold and grown in the same medium and conditions to midexponential phase.

Isolation of Yeast Mitochondria

Yeast spheroplasts prepared by enzymatic digestion with lyticase were homogenized, and mitochondria were isolated by differential centrifugation (Guérin *et al.*, 1979).

Yeast cells were harvested by centrifugation at 2500*g* for 5 min at 20–25°C, resuspended in Milli-Q water and recentrifuged. The wet weight of the pelleted cells was used as the basis for subsequent resuspensions. Cells were resuspended to 0.1 g/mL in buffer containing 100 mM

Table I. The 35 Yeast Mitochondrial Carriers

Gene	Name	MIPS[a]	No. of amino acids	Transport function[b]	Knockout constructed?	Growth on lactate?[c]	Refs.
IIc4	AAC2	YBL030c	318	ADP/ATP	No	(Petite)	Lawson and Douglas (1988)
IIc5	AAC3	YBL045w	308	ADP/ATP	No	—	Kolarov et al. (1990)
XIIIc3	AAC1	YMR056c	309	ADP/ATP	No	—	Adrian et al. (1986)
XVc3	ODC2	YOR222w	307	Oxodicarboxylate	Yes	Yes	Palmieri et al. (2001a)
XVIc1	ODC1	YPL134c	310	Oxodicarboxylate	Yes	Yes	Palmieri et al. (2001a)
IIc3	CTP1	YBR291c	299	Citrate	No	—	Kaplan et al. (1995)
Xc2	SFC1	YJR095w	322	Succinate/fumarate	Yes	Yes	Palmieri et al. (1997a)
XVc1	CRC1	YOR100c	327	Carnitine	Yes	Yes	Palmieri et al. (1999a)
XVc2	ARG11	YOR130c	292	Ornithine/arginine	No	—	Crabeel et al. (1996); Palmieri et al. (1997b)
IXc2	FLX1	YIL134w	311	Flavin	Yes	No	Tzagoloff et al. (1996)
XIc1	OAC1	YKL120w	324	Oxaloacetate/sulphate	Yes	Yes	Palmieri et al. (1999b)
XIIc1	DIC1	YLR348c	298	Dicarboxylate	Yes	Yes	Kakhniashvili et al. (1997); Palmieri et al. (1996)
XVIc5	ANT1	YPR128c	328	Peroxisomal ATP/AMP	Yes	Yes	Palmieri et al. (2001b)
Xc1	MIR1	YJR077c	311	Phosphate	No	(Petite)	Murakami et al. (1990); Phelps et al. (1991)
Vc1		YER053c	300	Phosphate	No	—	Nelson et al. (1998)
XIIIc2	YHM2	YMR241w	314	Tricarboxylate	Yes	Yes	Mayor et al. (1997)
XVIc4		YPR011c	326		Yes	Yes	
VIIc1		YGR096w	314		Yes	Yes	
VIIIc1	LEU5	YHR002w	357	(Coenzyme A)	Yes	No	Prohl et al. (2001)
XIVc1		YNL083w	545	(Aspartate/glutamate)	Yes	Yes	del Arco et al. (2000); Palmieri et al. (2001c)
XVIc3		YPR021c	902	(Aspartate/glutamate)	Yes	Yes	del Arco et al. (2000); Palmieri et al. (2001c)
XVIc2	YMC1	YPR058w	307		Yes	Yes	
IIc1	YMC2	YBL104w	329		Yes	Yes	
Xc3	MRS3	YJL133w	296	(mtRNA splicing)	Yes	Yes	Wiesenberger et al. (1991)
XIc2	MRS4	YKR052c	296	(mtRNA splicing)	Yes	Yes	Wiesenberger et al. (1991)
XIVc2	PET8	YNL003c	284		Yes	No	
XIIIc1		YMR166c	368		Yes	Yes	
VIIc2		YGR257c	366		Yes	No	
Vc2		YEL006w	335		Yes	Yes	
IXc1		YIL006w	373		Yes	Yes	
IIc2	RIM2	YBL192w	377		No	(Petite)	van Dyck et al. (1995)
IVc1		YDL119c	307		Yes	Yes	
IVc2	YHM1	YDL198c	300		Yes	No	
VIc1		YFR045w	309		Yes	Yes	
IVc3		YDR470c	502		Yes	No	

Note. Genes of known function are listed in the top half of the table according to the relatedness of the protein sequences as in Nelson et al. (1998).

[a] http://www.mips.biochem.mpg.de/.

[b] Unsubstantiated or nontransport functions are in parentheses.

[c] Results in parentheses are from the cited references.

Tris/HC1 and 20 mM dithiothreitol, pH 9.3, incubated with gentle shaking for 10 min at 30°C, collected by centrifugation at 2500g for 5 min, washed twice in buffer containing 100 mM Tris/HCl and 500 mM KCl, pH 7.0, and resuspended to 1 g/mL in isotonic spheroplasting buffer (40 mM citric acid, 120 mM Na$_2$HPO$_4$, 1.35 M sorbitol, 1 mM EGTA, pH 5.8). Lyticase was added at 3 mg/g cells, and the cells were incubated at 30°C for 15–30 min with gentle shaking. Spheroplast formation was monitored by

diluting small aliquots 50-fold with water or with isotonic spheroplasting buffer, and comparing A$_{600}$.

When 80–90% of the cells were converted to spheroplasts, they were centrifuged for 5 min at 2500g at 4°C. Subsequent steps were at 4°C. Spheroplasts were washed twice in buffer containing 10 mM Tris/maleate, 750 mM sorbitol, 400 mM mannitol, 2 mM EGTA, and 0.1% BSA, pH 6.8, and then resuspended in mitochondrial isolation buffer (600 mM mannitol, 10 mM Tris/maleate, 0.5 mM

Na_2HPO_4, 2 mM EGTA, 1 mM EDTA, and 2% BSA, pH 6.8), with one protease inhibitor tablet (Complete®, Boehringer) added per 40 mL immediately before use. They were homogenized by 12 passes with a Wesley Coe homogenizer. The homogenate was centrifuged at $800g$ for 10 min. The supernatants were removed by pipette, to prevent disruption of the pellet, recentrifuged at $800g$ for 10 min, and then centrifuged at $11000g$ for 10 min. Mitochondrial pellets were washed in buffer containing 10 mM Tris/maleate, 650 mM mannitol, and 2 mM EGTA, pH 6.8, and then resuspended in a small volume of this buffer and assayed for protein content by the biuret method (Gornall *et al.*, 1949).

Proton Leak Measurements

Respiration rate and membrane potential were measured simultaneously at 30°C using electrodes sensitive to oxygen and to the potential-dependent probe methyltriphenylphosphonium ($TPMP^+$) (Stuart *et al.*, 2001b). Yeast or mammalian mitochondria were suspended at 0.5 mg of protein/mL in buffer containing 20 mM Tris/HCl, 650 mM mannitol, 0.5 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4, and 0.2% BSA, pH 6.8. Oligomycin (l μg/mL), nigericin (100 ng/mL), myxothiazol (3 μM), and ascorbate (2 mM) were added. The TPMP electrode was then calibrated with four sequential 1 μM additions up to 4 μM TPMP. Ascorbate oxidation was increased progressively by adding N,N,N′,N′-tetramethyl-1-*p*-phenylenediamine (TMPD) up to 62.5 μM. After each run, 0.2 μM FCCP was added to release TPMP for baseline correction. Membrane potentials were calculated as described by Brand (1995), assuming a TPMP binding correction of 0.35 (μL·mg protein)$^{-1}$.

Statistical Analyses

Analysis of variance (ANOVA) and Student's *t* test were used for statistical calculations.

RESULTS

Twenty-seven of the 35 genes for mitochondrial carriers were deleted in yeast (Table I), then each knockout strain was grown on selective lactate medium. Six knockouts did not grow on nonfermentable substrates, and so could not be used to isolate mitochondria. Inactivation of the *IIc2* (*RIM2*) gene causes a petite phenotype (van Dyck *et al.*, 1995), and so it was not used in this study. We did not test the remaining seven genes because they already had well-characterized functions when this study was initiated (the transport of adenine nucleotides (three isoforms),

phosphate (two isoforms), citrate, and ornithine). In any case, deletion of genes encoding the ADP/ATP (*IIc4*) and phosphate carriers (*Xcl*) results in cells unable to grow on a nonfermentable carbon source (Lawson and Douglas, 1988; Murakami *et al.*, 1990).

The effect on growth rate of deleting each of the remaining 21 carriers was assessed by measuring doubling time in liquid medium, using DL-lactic acid as the sole carbon source. Figure 1 shows that deletion of *Xc2* (*SFC1/ACR1*), which encodes the succinate/fumarate antiporter, strongly showed growth, giving a doubling time twice as high as wild-type YPH499 cells (8.5 ± 0.6 h vs. 4.2 ± 0.5 h). The doubling time of *S. cerevisiae* cells lacking either *XVIc1* (*ODC1*, encoding one of the two oxodicarboxylate carriers) or *XIc2* (*MRS4*) was slightly, but significantly, increased (by 30 and 20%, respectively). Deletion of the other carrier genes did not significantly affect the growth rate of *S. cerevisiae* cells on DL-lactic acid. Removal of a putative endogenous uncoupling protein might be expected to decrease doubling time by improving the efficiency of oxidative phosphorylation, but secondary effects might obscure the picture.

Respiration rates (and proton conductances, see below) with ascorbate/TMPD as substrate were measured on mitochondria isolated on the same day from two or three knockouts and from the wild-type strain (Table II). This substrate was chosen because TMPD feeds electrons directly to cytochrome *c* for oxidation by cytochrome oxidase, and so electron supply should be unaffected by the presence or absence of mitochondrial substrate carriers. To aid comparisons, rates for mitochondria from each knockout were expressed as a percentage of the rate for the control wild-type mitochondria prepared on the same day. In general, deletion of each of the mitochondrial carriers did not change the nonphosphorylating respiration rate, suggesting that they did not contribute to the basal proton conductance, which has high control over respiration rate under these conditions (Brand *et al.*, 1988). Similarly, deletion of carriers did not generally change the rate uncoupled with FCCP, which is limited by the activity of the electron transport chain. There were five significant exceptions. Deletion of the genes *Xc2*, *XIcl*, *XIIc1*, and *IIcl* significantly increased nonphosphorylating respiration, which could suggest that deleting these genes resulted in an increase mitochondrial proton conductance. Deletion of *XVIcl* (encoding one of the oxodicarboxylate carriers) significantly decreased oxygen consumption to about 75% of control levels, which could suggest that deleting *XVIc1* resulted in a decrease in mitochondrial proton conductance, possibly because it is a yeast uncoupling protein.

Nonphosphorylating respiration rate is a poor indicator of mitochondrial proton conductance because it is
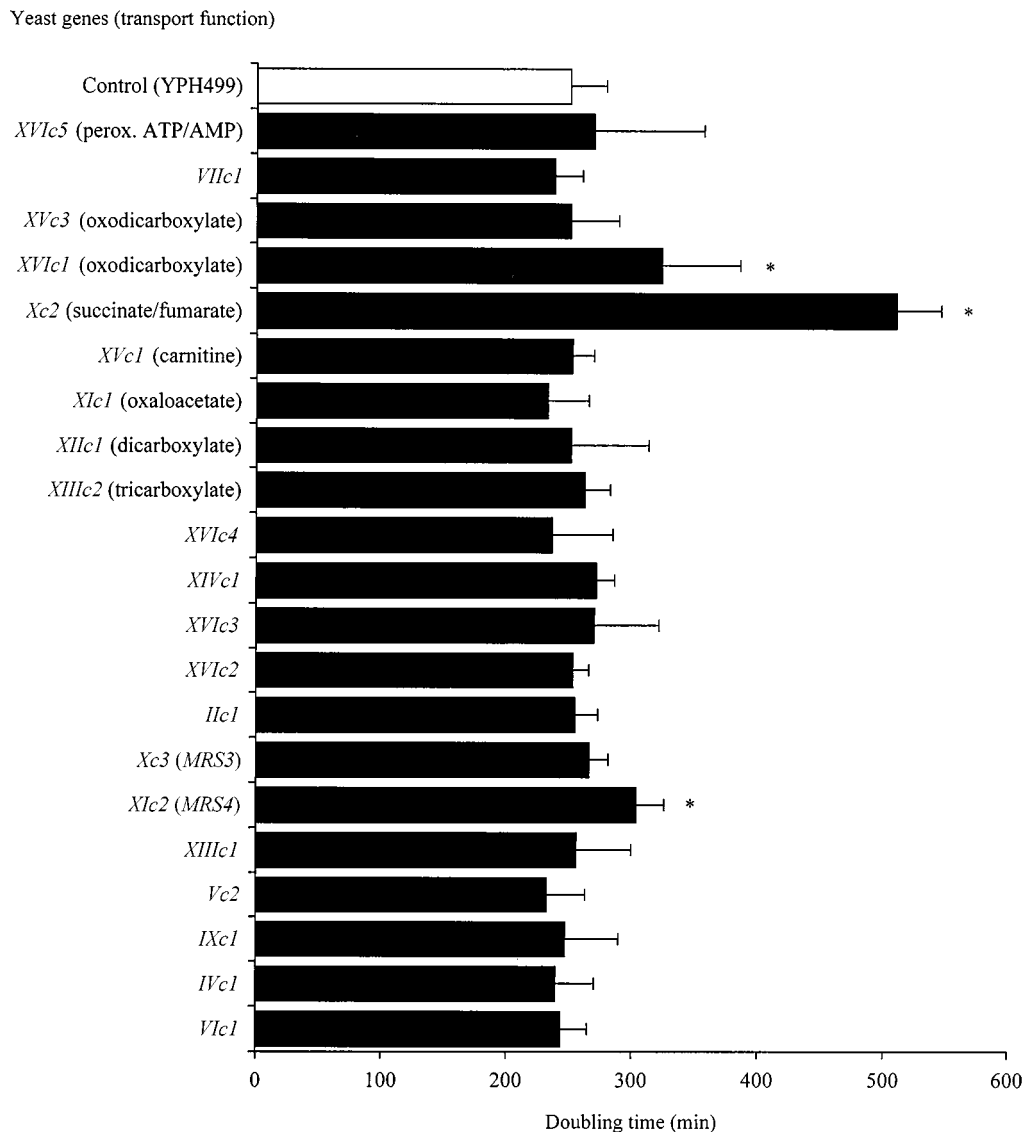
Yeast genes (transport function)

Fig. 1. Effect of mitochondrial carrier gene deletion on yeast growth. Cells were grown aerobically at 30°C in selective yeast nitrogen base media containing 2% DL-lactic acid as sole carbon source. Open bar: wild-type YPH499 cells; closed bars: knockout mutant cells. Values are mean ± SEM ($n = 2$–6). Perox., peroxisomal. *, significantly different from wild-type ($p < 0.05$).

influenced not only by proton conductance but also by substrate oxidation: A decrease in respiration could be caused by deletion of a putative uncoupling protein, but equally, it could be caused by inhibition of the electron transport chain (in this case, cytochrome c or cytochrome oxidase). In the same way, an unchanged respiration rate might hide a decrease in proton conductance that was opposed by an increase in electron transport chain activity. For this reason, the proton leak kinetics were determined directly in mitochondria isolated from wild-type and knockout yeast. Figure 2 shows the dependence of proton leak rate on mito-

chondrial membrane potential for 25 mitochondrial preparations from wild-type YPH499 yeast, compared to the kinetics of proton leak in mitochondria isolated from various rat tissues, and measured under closely similar experimental conditions. The basal proton conductance was higher in yeast mitochondria than in rat liver and kidney mitochondria, confirming observations in Brand *et al.* (1999), but was similar to the conductance measured in rat skeletal muscle mitochondria. The downward displacement of the curves for rat liver or kidney seen in Fig. 2 compared with yeast or rat skeletal muscle illustrates how a deletion

Table II. Respiration Rates of Mitochondria Isolated From Knockout Mutant Yeast and Control Wild-Type Yeast

Gene	Transport function	n	Ascorbate/TMPD[a]	% of paired control	Ascorbate/ TMPD + FCCP[a]	% of paired control
Wild-type						
YPH499 control strain		25	358 ± 17	100	499 ± 25	100
XVIc5	Peroxisomal ATP/AMP	2	409 ± 55	102 ± 14	557 ± 90	103 ± 21
VIIc1		3	411 ± 53	113 ± 8	614 ± 82	116 ± 7
XVc3	Oxodicarboxylate	4	380 ± 26	108 ± 6	553 ± 41	110 ± 7
XVIc1	Oxodicarboxylate	3	270 ± 20	$76 \pm 2^*$	369 ± 21	$73 \pm 2^*$
Xc2	Succinate/fumarate	2	423 ± 3	$148 \pm 41^*$	608 ± 21	$147 \pm 38^*$
XVc1	Carnitine	3	411 ± 17	106 ± 17	549 ± 30	105 ± 19
XIc1	Oxaloacetate/sulphate	2	300 ± 40	$123 \pm 12^*$	476 ± 57	$133 \pm 6^*$
XIIc1	Dicarboxylate	2	426 ± 16	$129 \pm 5^*$	564 ± 19	$129 \pm 4^*$
XIIIc2	Tricarboxylate	3	325 ± 42	89 ± 6	462 ± 52	92 ± 7
XVIc4		4	411 ± 21	106 ± 3	559 ± 24	108 ± 5
XIVc1	(Aspartate/glutamate)	3	358 ± 28	91 ± 11	465 ± 44	88 ± 14
XVIc3	(Aspartate/glutamate)	3	413 ± 28	102 ± 10	596 ± 54	104 ± 13
XVIc2		2	287 ± 22	105 ± 11	394 ± 51	110 ± 12
IIc1		2	331 ± 7	$121 \pm 6^*$	448 ± 3	$125 \pm 4^*$
Xc3	(mtRNA splicing)	3	409 ± 12	101 ± 8	611 ± 22	107 ± 9
XIc2	(mtRNA splicing)	3	344 ± 24	99 ± 7	463 ± 36	99 ± 8
XIIIc1		3	317 ± 51	120 ± 27	450 ± 66	118 ± 24
Vc2		3	369 ± 64	104 ± 13	526 ± 96	104 ± 14
IXc1		3	341 ± 73	94 ± 8	424 ± 107	81 ± 4
IVc1		2	440 ± 27	124 ± 29	561 ± 62	116 ± 29
VIc1		1	279	89	405	92

Note. Values are mean $\pm$ SEM from n independent mitochondrial preparations. Ascorbate (2 mM) and TMPD (62.5 μM) were added as respiratory substrates. Rates were measured after addition of TMPD, and again after addition of 0.2 μM FCCP.
[a] Nanomole of O per min per mg of mitochondrial protein.
* $p < 0.05$, significantly different from paired control.

of a yeast mitochondrial carrier should affect the proton leak kinetics if it catalyzed basal proton conductance. At a given membrane potential, the proton leak rate in the knockout mitochondria would be lower than that in wild-type mitochondria, regardless of any effects on the electron transport chain, which would only affect the maximum rate and potential that could be achieved.

Figures 3–5 show the effects of deletion of 21 yeast mitochondrial carriers on mitochondrial proton leak kinetics. Mitochondria from 19 of the knockout strains had proton leak kinetics that were indistinguishable from the proton leak kinetics of mitochondria from wild-type yeast, showing that none of these 19 carriers were responsible for basal proton conductance in yeast mitochondria. In particular, the dicarboxylate carrier (DIC1) encoded by *XIIc1* is the yeast transporter most similar to the mammalian and plant UCPs (Brand *et al.*, 1999), but deletion of this gene clearly had no effect on the basal mitochondrial proton conductance (Fig. 3(h)).

Mitochondria isolated from *S. cerevisiae* lacking the genes *XVIc1* (Fig. 3(d)) and *Xc2* (Fig. 3(e)) had proton leak kinetics that may have differed from wild-type mitochondria. Mitochondria from yeast lacking *XVIc1*

(Fig. 3(d)) had a greater rate of proton leak than controls at any membrane potential above about 90 mV, suggesting greater proton conductance in the absence of this oxodicarboxylate carrier. The maximum respiration rate achieved was less than controls, showing that deletion of the carrier had a second effect—a decrease in the activity of the electron transport chain (cytochrome *c* or cytochrome oxidase). These effects can explain the growth and respiration phenotypes of the *XVIc1* deletion: slower growth (Fig. 1) could be caused by compromised energy metabolism because of the combination of uncoupling and inhibition of electron transport; while decrease in mitochondrial respiration rate to 76% of control (Table II) would not be due to decreased endogenous uncoupling, but the result of electron transport inhibition, overcoming the increased respiration rate expected from uncoupling. This interpretation is supported by the observation in Table II that respiration in the presence of FCCP was also inhibited to 74% of control, showing a decrease in the maximum rate of electron transport under conditions in which endogenous proton conductance has no role. It is clear that expression of *XVIc1* does not cause the basal proton conductance of yeast mitochondria, but deletion of this oxodicarboxylate
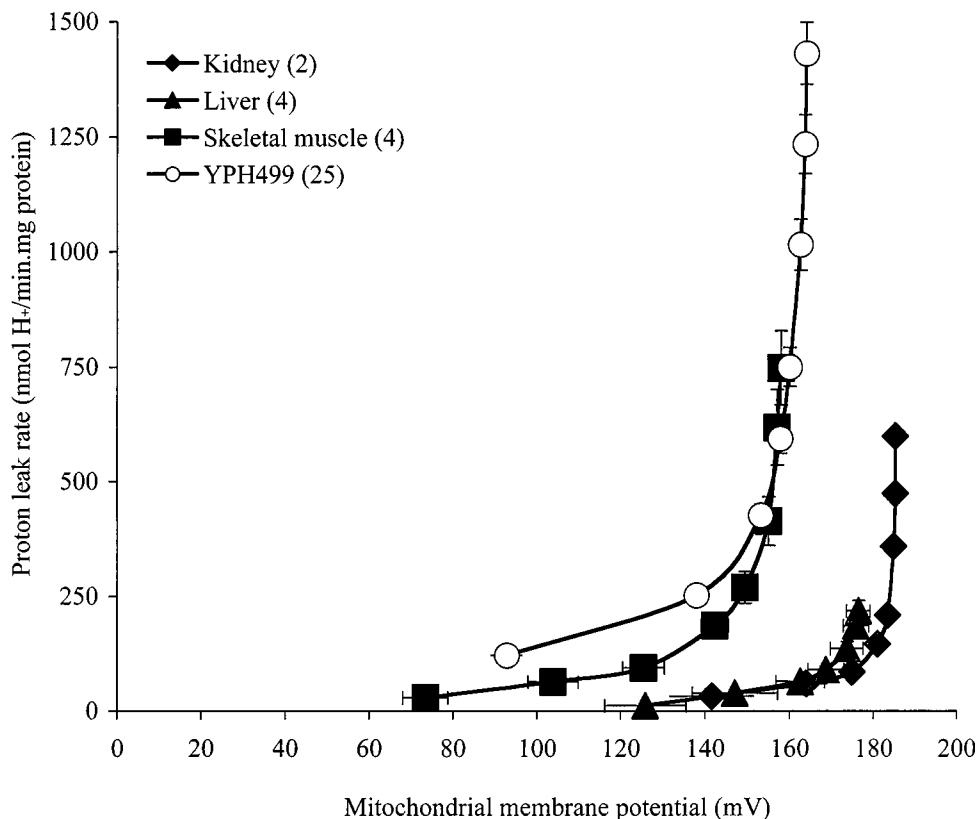
Fig. 2. Kinetics of proton leak in mitochondria isolated from wild-type YPH499 yeast and from various rat tissues. Values are mean $\pm$ SEM from (n) independent mitochondrial preparations. Mammalian mitochondria were isolated as described in Echtay *et al.* (2002). Proton leak rate was calculated from nonphosphorylating respiration rate assuming a constant stoichiometry of 4 H^+/O for oxidation of ascorbate/TMPD.

carrier has some secondary deleterious effect on yeast mitochondria.

Mitochondria from yeast lacking *Xc2* (Fig. 3(e)) may have had a lower rate of proton leak than controls at any membrane potential above about 100 mV, suggesting lower proton conductance in the absence of the succinate/fumarate carrier. The maximum respiration rate achieved was greater than controls, showing that deletion of the carrier had a second effect—an increase in the activity of the electron transport chain (cytochrome *c* or cytochrome oxidase). These effects can explain the respiration phenotype of the *Xc2* deletion: The increase in mitochondrial respiration rate to 148% of control (Table II) would not be due to increased endogenous uncoupling, but the result of electron transport stimulation, overcoming the decreased respiration rate expected from tighter coupling. This interpretation is supported by the observation in Table II that respiration in the presence of FCCP was also stimulated to 147% of control, showing an increase in the maximum rate of electron transport under conditions in which endogenous proton conductance has no role.

The slower growth (Fig. 1) may be due to a requirement for succinate/fumarate transport in vivo (Palmieri *et al.*, 1997a), as decreased proton conductance and increased electron transport chain activity might be expected to increase the growth rate of yeast cells. It is clear that deletion of *Xc2* has multiple effects on yeast cells. The slower growth rate may alter the cell wall and cause changes in mitochondrial damage during isolation which in turn change the measured proton conductance, but we cannot exclude the possibility that the succinate/fumarate carrier does make a small contribution to the basal proton conductance of yeast mitochondria.

DISCUSSION

In this paper we measure the basal proton conductance of yeast mitochondria. ATP induces proton conductance (Prieto *et al.*, 1992) in different laboratory yeast strains, including YPH499 (D. Roussel and M.D. Brand, unpublished). It also opens an unselective anion channel
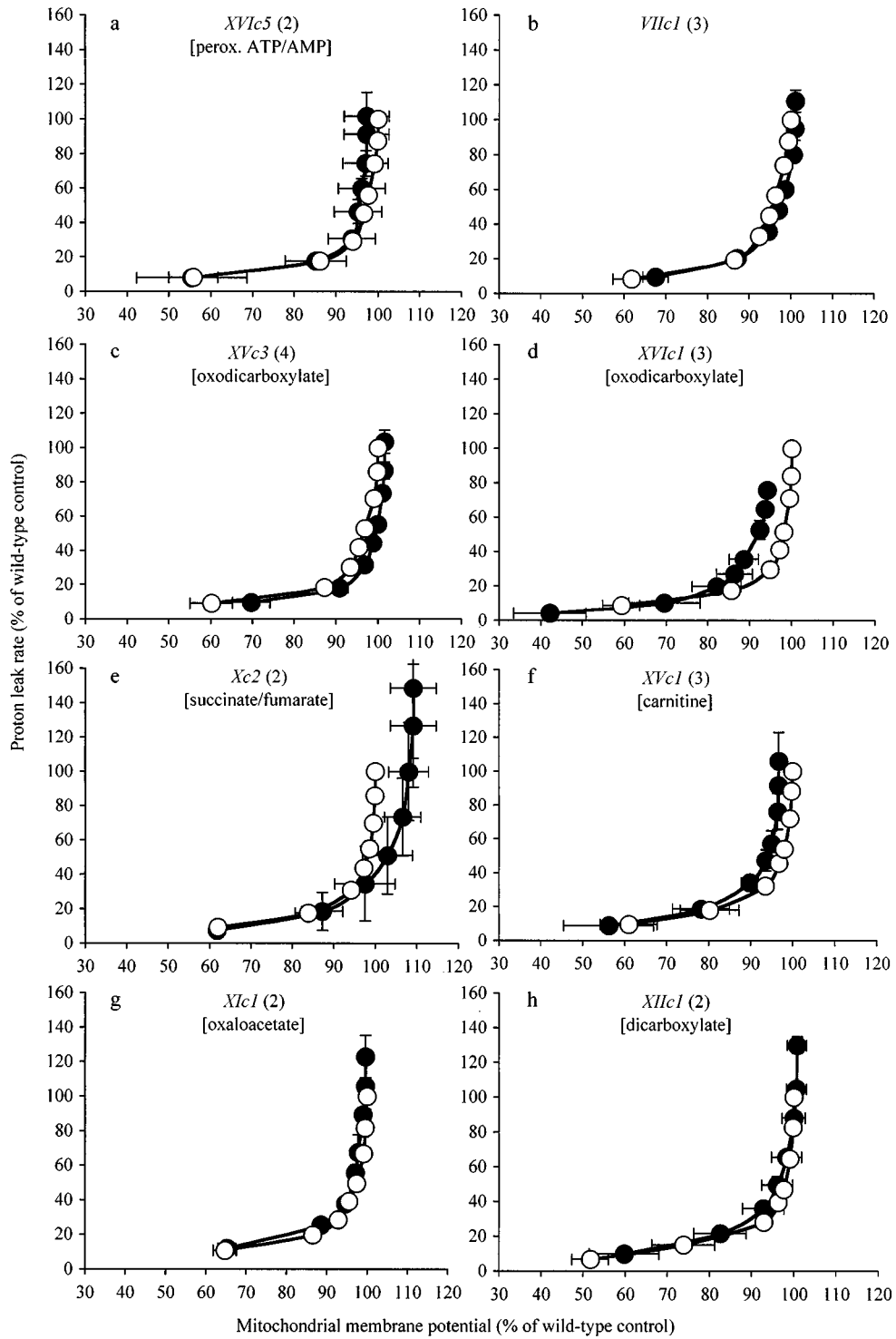
Fig. 3. Kinetics of proton leak in mitochondria isolated from knockout mutant yeast (●) and paired control wild-type yeast (○). The deleted gene is specified in each graph, for (*n*) independent mitochondrial preparations. Proton leak rates and mitochondrial membrane potentials have been normalized to paired control values. Perox., peroxisomal.
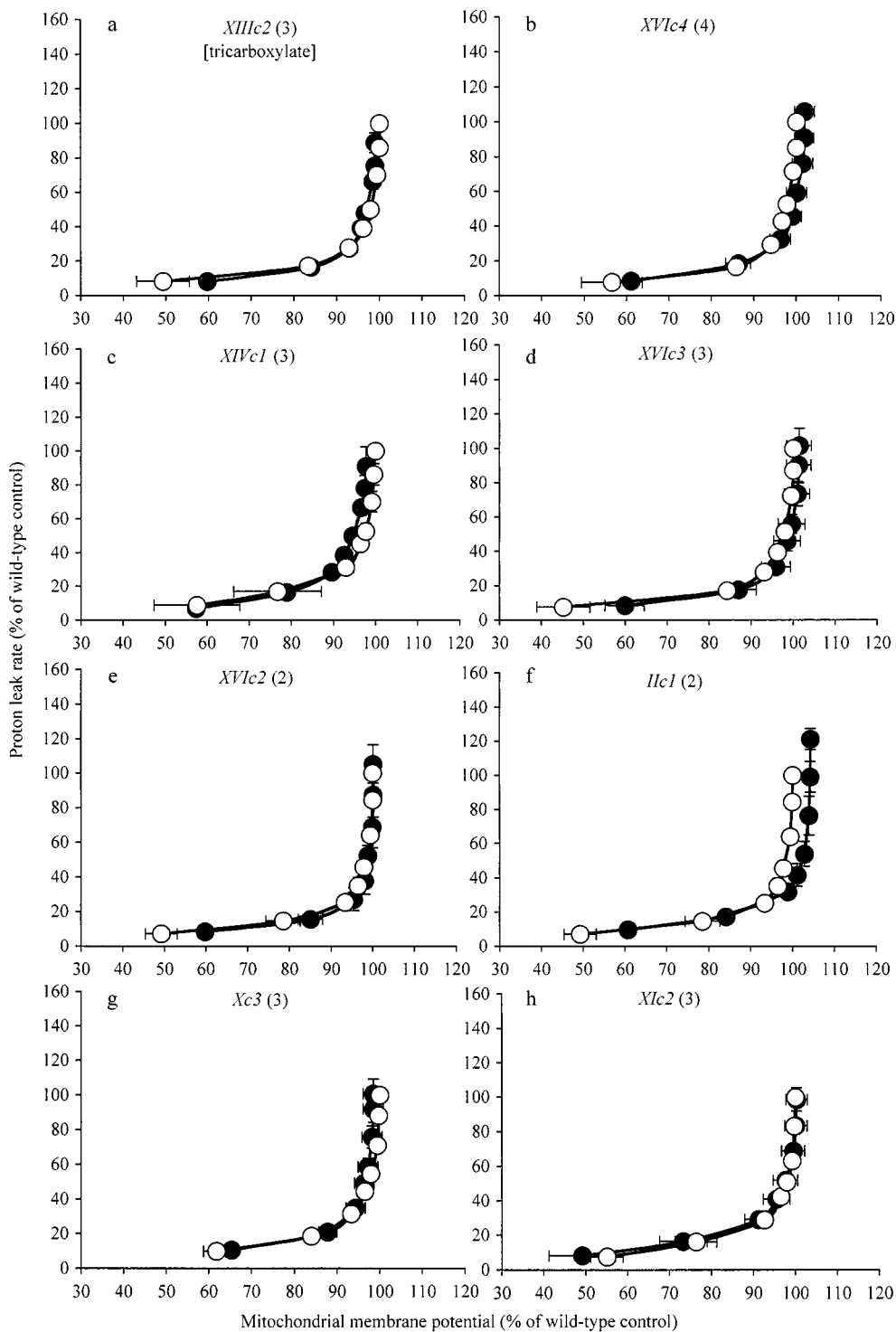
Fig. 4. Kinetics of proton leak in mitochondria isolated from knockout mutant yeast (●) and paired control wild-type yeast (○). For details, see Fig. 3.
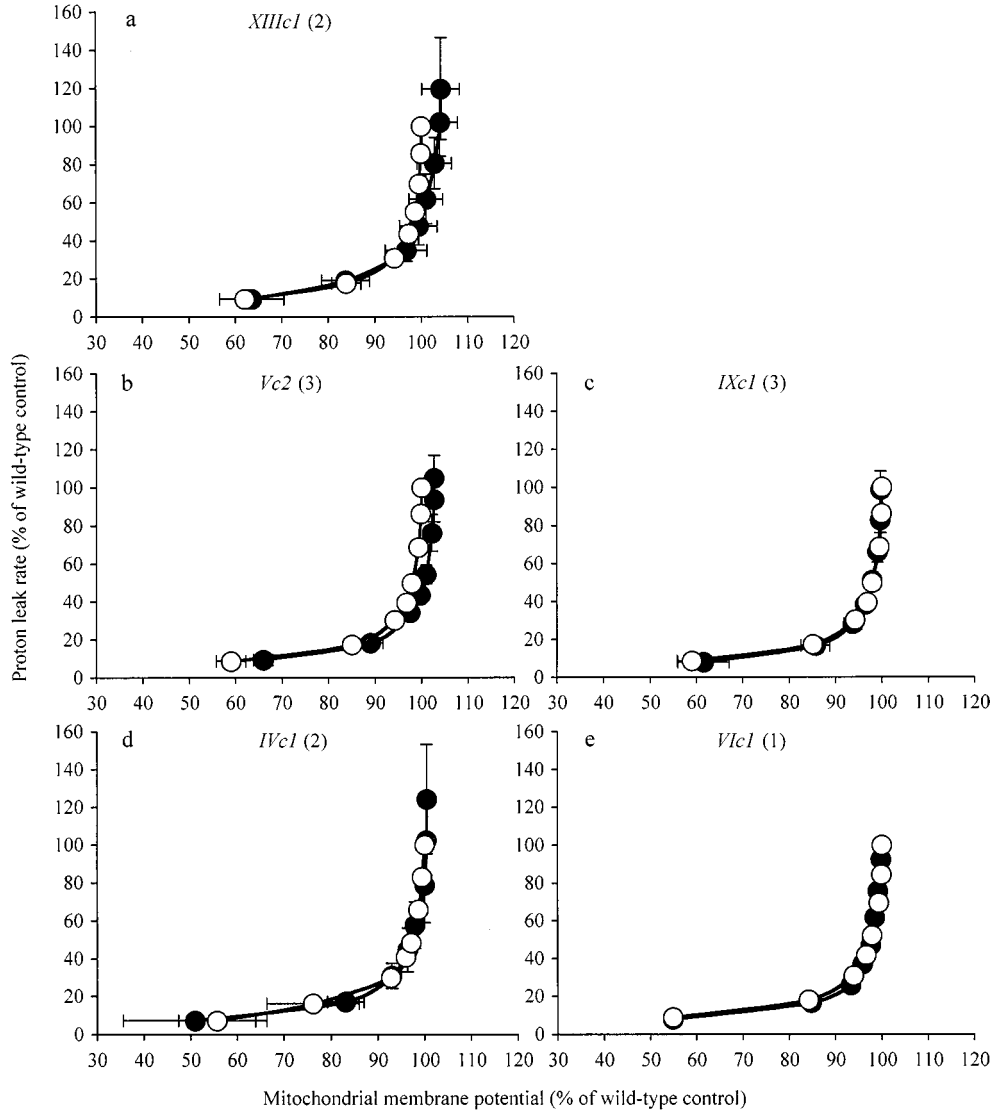
Fig. 5. Kinetics of proton leak in mitochondria isolated from knockout mutant yeast (●) and paired control wild-type yeast (○). For details, see Fig. 3.

(Guérin *et al.*, 1994) and induces an electrophoretic potassium pathway (Roucou *et al.*, 1995). These ATP-inducible ion permeabilities could interfere with measurement of basal proton conductance, and so they were inhibited in our experiments by omitting ATP, adding oligomycin (to prevent ATP synthesis), and working in a low salt medium containing mannitol and supplemented with 10 mM phosphate and 5 mM magnesium. Bovine serum albumin (0.2%) was added to prevent fatty acid uncoupling, which makes up 15–30% of total leak in freshly prepared liver mitochondria (Brown and Brand, 1991). There are no clear homologues of mammalian or plant UCPs in the genome of *S. cerevisiae* (Brand *et al.*,

1999). Hence, the relatively high basal proton conductance of yeast mitochondria (Fig. 2) is not caused by known pathways.

In this paper we explore the hypothesis that the basal proton conductance in yeast (and perhaps all) mitochondria is caused by specific members of the mitochondrial carrier protein family. The results in Figs. 3–5 show that 20 of the 35 members of the family that are present in yeast do not contribute significantly to basal proton leak, as deleting them does not reduce proton conductance. We cannot exclude the possibility that the succinate/fumarate carrier encoded by *Xc2* makes some contribution, but it does not seem to be large.

Deletion of six of the carriers prevented growth on lactate (Table I), and deletion of at least two others (*IIc4* and *Xc1*) also prevents growth on nonfermentable carbon sources (Lawson and Douglas, 1988; Murakami *et al.*, 1990). Yeast grown on fermentable carbon sources do not make sufficient amounts of coupled mitochondria, and so we could not test whether deletion of these genes altered mitochondrial proton conductance. It is possible that basal proton conductance is catalyzed by one or more of these eight carriers, and that proton conductance is essential for growth (for example, by restricting harmful superoxide production) (Papa and Skulachev, 1997; Brand, 2000). This could be one explanation for the lack of growth on nonfermentable carbon sources. Other approaches will be needed to answer these questions.

Although individual deletion of 20 of the mitochondrial carriers had no measurable effect on mitochondrial proton conductance, their aggregate activity might still contribute towards basal proton conductance in yeast mitochondria. If this was true, deletion of one carrier would not alter the measured proton conductance unless the protein was abundant. The Codon Adaptation Index (CAI) may indicate a protein's abundance; highly expressed yeast genes have CAI values greater than 0.5. From the CAI values given in Nelson *et al.* (1998) for the yeast mitochondrial carrier genes, it appears that *IIc4*, which encodes an isoform of the ADP/ATP carrier (AAC2), is the only highly expressed carrier in yeast, suggesting that it is the most abundant carrier protein of the yeast mitochondrial inner membrane. Unfortunately, disruption of this gene causes a petite phenotype, making the disruption strategy ineffective in studying the role AAC2 protein could play in mitochondrial proton permeability.

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