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Characterization of the *Drosophila melanogaster* Mitochondrial Proteome

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We have combined high-resolution two-dimensional (2-D) gel electrophoresis with mass spectrometry with the aim of identifying proteins represented in the 2-D gel database of *Drosophila melanogaster* mitochondria. First, we purified mitochondria from third instar *Drosophila* larvae and constructed a high-resolution 2-D gel database containing 231 silver-stained polypeptides. Next, we carried out preparative 2-D PAGE to isolate some of the polypeptides and characterize them by MALDI-TOF analysis. Using this strategy, we identified 66 mitochondrial spots in the database, and in each case confirmed their identity by MALDI-TOF/TOF analysis. In addition, we generated antibodies against two of the mitochondrial proteins as tools for characterizing the organelle.

Keywords: *Drosophila* • mitochondria • proteomics • 2D gels • MALDI-TOF

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Introduction

Since the early 90s, cataloguing the proteins of single-celled microorganisms, cells, biological fluids, tissue, and whole organisms has been undertaken at a rapid pace as advances are made in protein and peptide separation, detection and identification. This progress is evident from the numerous databases that are available (see for example the directory of 2D databases on the ExPASy server at http://us.expasy.org/ch2d/). It has been supported by continual innovation in 2D gel electrophoresis technology and in methods of identification, and the availability of complete genome sequences.

These techniques, however, are not free of limitations, at least for complex eukaryotes where the number of proteins in a single cell is estimated to approach 10 000.[1] This means that proteome analysis on whole cell extracts tends to miss low-abundance proteins. One solution to this problem is to take advantage of the compartmentalization of the eukaryotic cell, and to analyze subcellular organelles. These can be isolated in good yield and the complexity of their protein composition is lower than that of whole cells. Classical cell subfractionation yields four major fractions: nucleus, mitochondria, microsomes and cytosol. Of these, the nucleus and mitochondria contain 5−10% of the total proteins and constitute interesting targets of analysis because they are discrete functional units.

Mitochondria are ubiquitous cytoplasmic organelles that play crucial roles in oxidative phosphorylation, β-oxidation of fatty acids and the Kreb's cycle. They also generate most of the cellular reactive oxygen species and are crucial for calcium signaling.[2] Given their importance in cellular physiology, it is not surprising that defective mitochondrial biogenesis and metabolism are involved in a broad range of pathologies. For example, the Human disease catalog lists about one hundred diseases related to mitochondrial dysfunction such as diabetes, obesity, cancer, aging, neurodegeneration, and cardiomyopathy.[3] However, many of the nuclear genes involved remain to be identified, and an inventory of such genes is essential for a better understanding of mitochondrial function. As a result there have been several recent efforts to define the mitochondrial proteome in humans and model organism. For example, the mitochondrial proteome of *Arabidopsis thaliana* was analyzed by 2D-PAGE, and 91 proteins were identified by peptide mass fingerprinting and MALDI-TOF mass spectrometry.[4] Similarly, Pflieger et al. [5] adopted a LC−MS/MS approach and identified 179 gene products in the yeast mitochondrion. This number was later increased to 750 by Sickmann et al.[6]

Drosophila is a well-established model organism in cellular and developmental biology, and, for the reasons adduced above as well as the need for markers of cellular organelles, we have initiated a project on the mitochondrial proteome. At present, the number of *Drosophila* nuclear genes that encode mitochondrial proteins and whose function has been studied by molecular genetic techniques, is relatively low, presumably because of the difficulty of isolating such genes in conventional genetic screens. In addition, mitochondrial alterations in *Drosophila* mimic some human diseases. For example mitochondrial dysfunction is the earliest manifestation of muscle degeneration and a prominent characteristic of individualizing spermatids in *parkin* mutants. Loss-of-function mutations in *parkin* result in an early onset form of programmed dead known as autosomal recessive juvenile Parkinsonism.[7]

We have compiled a catalog of the mitochondrial proteome using highly purified mitochondria from *Drosophila* third instar larvae. This provides a basis for identifying novel proteins or

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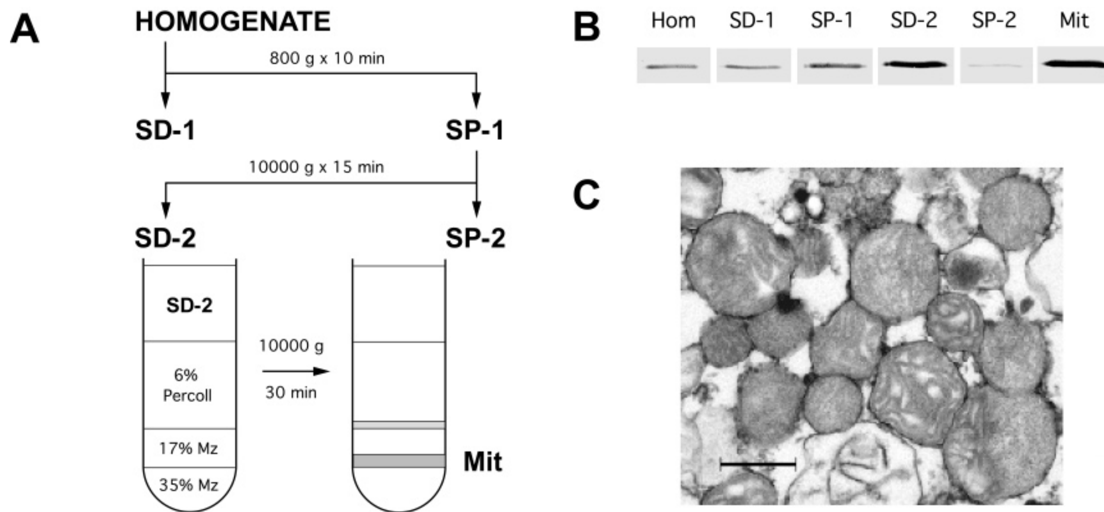

Figure 1. (A) Diagram of the hybrid Percoll/metrizamide discontinuous density gradient used to isolate mitochondria from *Drosophila melanogaster* third instar larvae. Mz, Metrizamide. (B) Western blot of samples of 100 micrograms of total protein from successive steps of the purification, using antibody against the mitochondrial matrix protein P1 precursor. (C) Morphology of the purified mitochondria. The preparation was fixed with 2.0% glutaraldehyde plus 2.0% tannic acid in 0.1 M cacodylate, pH 7.4, for 1 h at room temperature, followed by 1.0% OsO_4 plus 1.0% potassium ferrocyanide for 1 h at 4 °C. Sections were post stained with uranyl acetate and lead citrate. The bar represents 1.0 μm.

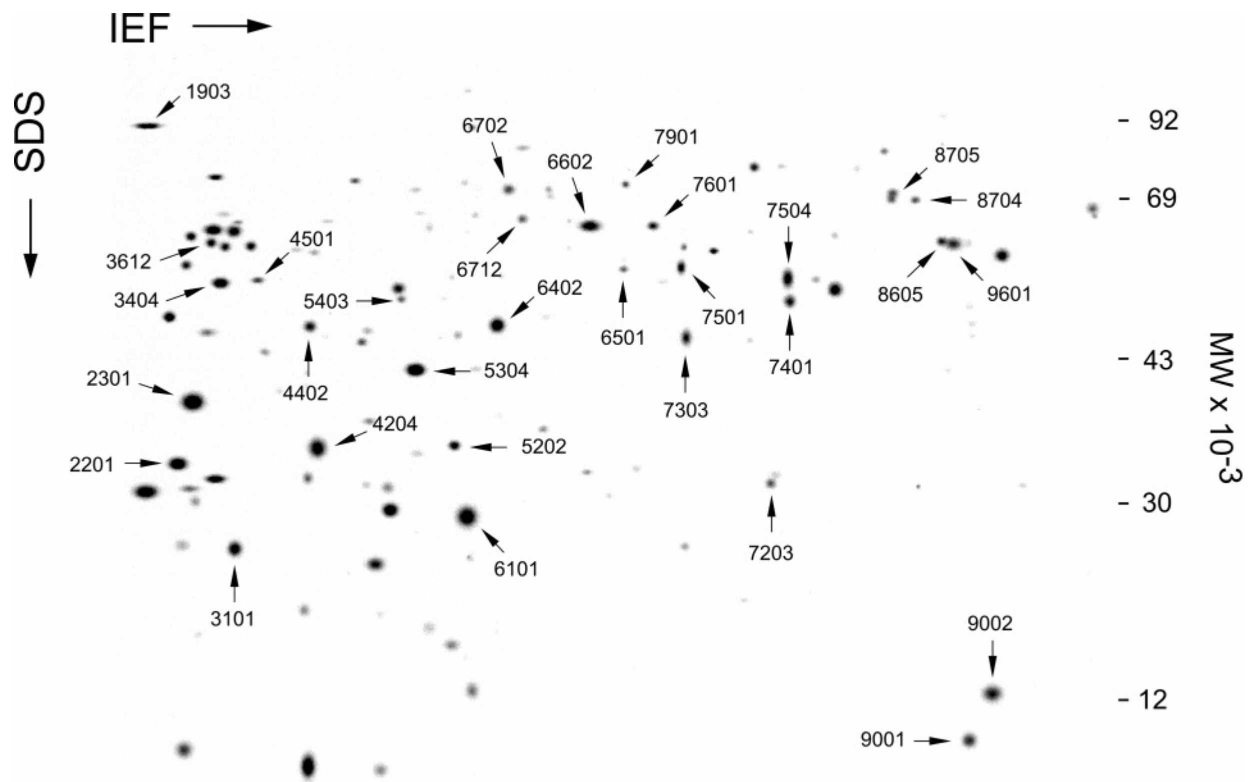

Figure 2. Two-dimensional gel of acidic (IEF) polypeptides from the mitochondria of *Drosophila melanogaster* third instar larvae. A total of 100 micrograms was applied per gel and 143 polypeptides were separated and quantified. The pH ranges from 7.0 (left) to 4.4 (right). Arrows indicate some of the identified polypeptides.

protein isoforms localized to the mitochondrion and can be used in future as a reference to examine the effects of mitochondrial dysfunction on the mitochondrial proteome. In addition, we have generated specific antibodies against two mitochondrial proteins that can be used as markers in a broad set of applications, or as a means of tracking the dynamics of mitochondria during normal or abnormal cellular and developmental processes.

Experimental Section

Procedures. 1. Fly Strains and Cell Culture. Laboratory stocks of wild-type *D. melanogaster* [strain Vallecas (Spain)] were used. We also employed the established Schneider cell line of *D. melanogaster*. These cells were grown in bottles (25 cm^2) in 20 mL of M3 medium supplemented with 5% foetal bovine serum.

Table 1. Identification by MALDI-TOF and MALDI-TOF-TOF MS of Acidic Polypeptides (IEF) Recorded in the *Drosophila* Mitochondria Database

SSP no.	KDa/p*I*	N[a]/%[b]	sequence determined by MS/MS	protein identified	fly base ID FBgn
1407	51.5/8.89	15/34.0	1299.77 ALGVLASLVWDR	l (1) G0030	0026708
			1315.64 EAGNNPSEEQLK	CG 3861	
			1439.81 SGQVVPGYGHAVLR		
			1688.90 ALVTETSVLDADEGIR		
1903	85.3/8.49	9/12.3	1457.81 IPFNVTPGSEQIR	ACONITASE	0010100
			1625.76 WVAVGDENYGEGSSR		
			1854.99 VLYSHLDDPANQDIVR		
2201	26.5/7.69	12/44.2	1135.58 NGFLLDGFPR	ADENYLATE KINASE−2	0022708
			1452.78 QTKPLVDYYGLR	Adk2	
			1601.77 SSDVFSTIDSIFQR		
			2039.07 YEPENIGINAILLGPPGSGK		
2301	31.0/7.69	8/30.1	1216.59 DFSVVYDLMK	CG 15093	0034390
			1329.66 DYAGGFSSALITK		
			1750.90 DTIFIDSSTISPDLVK		
			1891.93 QGLDANVFAEIINSSTGR		
2405	51.5/8.89	13/30.6	1299.74 ALGVLASLVWDR	CG 3861-PA	0026708
3101	24.8/8.88	8/33.5	830.53 GKPLFIR	RIESKE IRON−SULFUR PROTEIN	0021906
			1019.51 TAAEIETER	RfeSP	
			2527.35 KGPAPLNLEVPTHEFPNEGLLVVG		
3404	50.4/7.15	8/20.3	777.43 FEALAAR	l(1)G0255	0028336
			1389.72 SQINFPIGGATER	CG 4094-PB	
			1600.90 IAELTSLPFVTAPNK		
			1791.96 VYELALGGTAVGTGLNTR		
3612	61.0/8.58	14/31.2	963.50 YNLGLDLR	GLUTAMATE DEHYDROGENASE	0001098
			1059.51 NLNHVSYGR	(Splice isoform C)	
			1232.62 IPVTPSESFQK		
			1746.89 TFIVQGFGNVGLHTTR		
			1932.89 ESNYHLLESVQESLER		
4101	18.9/6.52	8/48.4	1008.51 ATENWFIK	Pdsw	0021967
			1031.48 QNWYHQR		
			1040.48 FEADQQFR		
			1315.70 MVDNEIVNILR		
4204	30.5/6.44	19/83.0	1461.73 LSLEGNFAPQSGNK	PORIN	0004363
			1505.77 VNNASQVGLGYQQK		
			1687.96 LRDGVTLTLSTLVDGK		
			1950.87 TSSGIEFNTAGHSNQESGK		
			2367.13 DFVLHTAVNDGQEFSGSIFQR		
			2534.28 WNTDNTLFTEVAVQDQLLEGLK		
4402	53.9/8.24	8/22	1590.87 ALDQLEAQVYILSK	ELONGATION FACTOR Tu	0024556
			1613.88 LLQEVDSFIPTPVR	MITOCHONDRIAL	
			1830.93 GITINVAHVEYQTETR		
			2186.17 ELDKPFLLPVENVYSIPGR		
4501	50.4/7.15	8/20.5	1257.63 TPVHPNDHVNK	l(1)G0255	0028336
5201	30.5/6.44	19/83.0	984.49 GYNFGLWK	PORIN	0004363
			1181.54 YQLDDDASVR		
			1505.77 VNNASQVGLGYQQK		
			2367.12 DFVLHTAVNDGQEFSGSIFQR		
5202	29.9/7.70	9/44.9	1295.66 DFPLSGYVELR	CG 12079	0035404
			1352.75 ILTDYGFEGHPQR		
			1532.74 VVCEPLELAQEFR		
			1753.82 FDLSAPWEQFPNFR		
5301	48.8/8.39	9/24.8	1203.58 YYTQMQTIR	l (1) G0334	0028325
			1530.75 GTIAYDIDHIQER	CG 7010-PC	
			1883.95 GDALPGIWVDGMDVLAVR		
			2474.18 SDAELGVSHLWTDVYSNNLEPK		
5304	39.3/7.66	8/29.3	1646.92 SIRPLDTAFIFASVR	CG 11876	0039635
			1801.90 VFILGEEVAQYDGAYK		
5401	44.0/7.07	11/25.4	1039.56 AFTGFIVER	CG 12262	0035811
			1174.58 LSAWEIDQGR		
			1273.65 KGDEWVINGQK		
			1463.84 TRPPVAAGAVGLAQR		
5403	46.4/6.29	19/50.0	1341.66 TVEAEAAHGTVTR	ISOCITRATE DEHYDROGENASE	0001248
6101	20.2/6.10	13/73.0	1372.77 LVPVAGLVDSFQK	ATP SYNTHASE, Subunit d	0016120
			1463.68 SKEQLEAEAQGHH		
			1545.79 IAQSSINWSALAER		
			1761.77 SLLPYDQMTMEDYR		
6402	38.5/6.96	9/31.6	1166.57 FPEIQFEEK	l (1) G0156	0027291
			1189.58 VAEYAFQYAK		
			1408.73 TLYDDVDVVTIR		
			1574.81 FGIPQAAIDSVNTNK		
6403	35.8/6.08	10/37.5	1165.58 DVDNIISAYR	l (1) G0334	0028325
			1530.75 GTIAYDIDHIQER		
			2474.19 SDAELGVSHLWTDVYSNNLEPK		

Table 1. (Continued)

SSP no.	KDa/p*I*	N[a]/%[b]	sequence determined by MS/MS	protein identified	fly base ID FBgn
6501	40.4/5.18	14/34.0	974.53 FDVPELVR 1590.75 IDFNQFEADIHNK 1790.89 VFSDAYLSDLEQLYK	NADH: UBIQUINONE REDUCTASE 42 KD SUBUNIT PRECURSOR	0019957
6602	57.0/6.37	8/19.2	1070.51 IYDEFVER 1428.78 EEIFGPVQQLIR 1563.71 TIPMDGDFFTYTR	CG 3752	0032114
6702	67.9/6.18	16/36.8	1296.65 HANGQITTADLR 1335.62 GEGGYLINGNGER 1473.84 LGANSLLDLVVFGR 1596.86 DHVYLQLHHLPPK 2054.02 NVIDTTLDNEVSTVPPAIR	SUCCINYL COENZIME A SYNTHETASE FLAVOPROTEIN SUBUNIT Scs-fp	0017539
6712	57.0/6.37	14/31.9	962.54 ANYIVGGLR 1070.51 IYDEFVER 1428.78 EEIFGPVQQLIR	CG 3752	0032114
7203	26.7/6.44	9/22.3	1283.71 VAEILQLPNMR 1297.72 GAMIPLLDLAQR 1611.87 RVEAILSIYPEGHK	CG 5703 − PA	0030853
7204	30.3/5.55	11/50.0	1349.66 SFGEAGDGLVELR 1485.71 IYTILGQDYDER 1617.85 AVVAQFDAGELITQR	l (2) 37Cc	0002031
7303	45.3/6.28	12/33.1	1097.53 YAAGFLNEGR 1266.73 LAQEQIQPLVK 1660.82 VPEENILGTFGHGYK	CG 3902	0036824
7304	45.3/6.28	10/41.3	1097.53 YAAGFLNEGR 1086.52 YYASEVAQR 1173.62 LQEQGVPFQK 1266.73 LAQEQIQPLVK 1660.82 VPEENILGTFGHGYK	CG 3902	0036824
7401	44.4/6.29	8/23.1	936.48 FSWGVANR 1291.71 AIYRDPFKPGK 1505.75 SQFLANSPNTALDK 1835.94 VQATYLWIDGTGENIR	GLUTAMINE SYNTHETASE 1	0001142
7501	52.9/6.21	12/36.8	1224.67 APGFAHLAALEK 1374.67 TYTQALPYFDR 1942.03 NLTLNFGPQHPAAHGVLR 2252.14 LFTQGYQVPPGATYTAIEAPK 2485.22 TEDIGIVTAEEALNYGFSGVMLR	CG 1970	0039909
7504	51.8/5.67	13/31.5	1134.63 IPLHELEQR 1290.73 RIPLHELEQR 1467.70 ADLTDYIQTHYK 2150.03 VASEDSGASTATVGLWIDAGSR 2460.19 SQTDLELEVENLGAHLNAYTSR	CG 3731	0038271
7505	51.8/5.67	15/36	1134.64 IPLHELEQR 1413.82 AVEILADIIQNSK 1467.73 ADLTDYIQTHYK 2150.05 VASEDSGASTATVGLWIDAGSR	CG 3731 − PA	0038271
7601	56.9/6.37	10/19.8	962.55 ANYIVGGLR 1070.52 IYDEFVER 1428.80 EEIFGPVQQLIR	CG 3752	0032114
7901	65.9/6.17	11/27.6	1187.56 DTFSFLTGSGR 1215.65 ITTHYTLNPR 1548.76 NPWLSPFQEFQR 1850.93 IPVDHNLALYEGPEQR	CG 12140	0033465
8201	24.5/5.81	9/42.0	993.47 RYPSGEER 1308.64 EPATINYPFEK	NADH: UBIQUINONE REDUCTASE 23 KD SUBUNIT PRECURSOR ND 23	0017567
8605	50.8/4.93	22/72.6	1439.78 VALTGLTVAEYFR	ATP SYNTHASE BETA CHAIN	0010217
8704	54.6/5.07	13/30.0	1609.89 AAVEEGIVPGGGTALLR	HEAT SHOCK PROTEIN 60 Hsp60	0015245
8705	54.6/5.07	17/41.7	1609.89 AAVEEGIVPGGGTALLR 1910.07 ISSVQSIIPALELANAQR 2038.16 KISSVQSIIPALELANAQR 2365.32 KPLVIIAEDIDGEALSTLVVNR	HEAT SHOCK PROTEIN 60 Hsp60	0015245
9001	16.6/5.55	6/47.7	984.59 VNDIALAIR 1074.52 EGIDGWEIR 2881.59 ITPTLQELGIPTIEELGYDKPELALK	CYTOCHROME C OXIDASE SUBUNIT Va	0019624
9002	16.7/5.73	7/73.9	1041.53 TFYDAAVVR 1482.84 AQAAIAVEVAEALVK 1665.88 QIDVPSFSGSFGILAK 2199.25 HVPTLAVLKPGVVQVVENDGK	l (1) G0230	0028342
9203	28.9/4.98	5/24.7	1008.49 GISQEFAEK 1303.72 SKPQFEVDIIK 1676.86 ELVEFLTEEIVAER 2098.08 LSDLATAHEHTSYIGLLEK	CG 6459	0034259

Table 1. (Continued)

SSP no.	KDa/p*I*	N[a]/%[b]	sequence determined by MS/MS	protein identified	fly base ID FBgn
9405	50.8/4.93	22/72	975.56 IGLFGGAGVGK 1439.77 VALTGLTVAEYFR 1601.79 VALVYGQMNEPPGAR 1921.94 DQEGQDVLLFIDNIFR 1988.02 AIAELGIYPAVDPLDSTSR 2135.05 SLQDIIAILGMDELSEEDK	ATP SYNTHASE BETA (FRAGMENT)	0010217
9601	50.8/4.93	22/67.7	1367.74 IINVIGEPIDER 1406.67 AHGGYSVFAGVGER 1439.78 VALTGLTVAEYFR 1677.92 LVLEVAQHLGENTVR 1921.95 DQEGQDVLLFIDNIFR 2252.06 IPSAVGYQPTLATDMGSMQER	ATP SYNTHASE BETA	0010217

[a] N = Number of matched peptides. [b] % = Coverage of full length protein by tryptic peptides.

2. Purification of Mitochondria. We adapted a method described for isolating mitochondria from Chinese hamster ovary cells.[8] In summary, *Drosophila* third instar larvae were homogenized in 0.25 M sucrose by four strokes with a Potter–Elvehjem homogeneizer. The homogenate was filtered through sterile gauze and centrifuged at 800 × *g* for 10 min. The supernatant was decanted and centrifuged again at 10 000 × *g* for 15 min. The mitochondrial pellet was resuspended in 0.25 M sucrose and centrifuged in a hybrid Percoll/metrizamide discontinuous gradient at 10 000 × *g* for 15 min. A band containing purified mitochondria was obtained at the metrizamide interface. The functional integrity of the purified mitochondria was measured by assaying the activity of respiratory chain enzymes and citrate synthase, as described.[9] Their structural integrity was assed by electron microscopy. The band of purified mitochondria was resuspended in PBS and centrifuged at 10 000 × *g* for 15 min. The pellet was fixed with 2.0% glutaraldehyde plus 2.0% tannic acid in 0.1 M cacodylate, pH 7.4, for 1 h at room temperature, followed by 1.0% OsO_4 plus 1.0% potassium ferrocyanide for 60 min at 4 °C. The sections were post stained with uranyl acetate and lead citrate and observed with a JEOL-1010 microscope operating at 80 kV.

3. Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was performed as described by O'Farrell [10,11] with some modifications [12]. Briefly, the first dimension for resolving acidic proteins (IEF) was performed on 230 × 2.3 mm 4% (w/v) polyacrylamide gels containing 2% ampholytes (1.6% pH 5–7; 0.4%, pH 3.5–10) at 1200 V for 20 h. Basic proteins (NEPHGE) were resolved on 150 × 2.3 mm 4% (w/v) polyacrylamide gels containing 2% ampholytes (1% pH 7–9; 1% pH 8–9.5) at 400 V for 4.5 h. The second dimension employed a 15% polyacrylamide gel (24 × 24 cm) run overnight at 18 °C. The gels were stained with Coomassie Brilliant Blue and silver nitrate [13].

4. Computer Analysis of 2D Patterns. The silver stained gels were digitized at 176 × 176 μm resolution with a pdi scanning densitometer, and the resulting 2-D images merged and analyzed with PDQUEST software (Version 5.1).

5. In-Gel Digestion. Coomassie- or silver-stained protein spots were excised and digested with trypsin (Promega, Madison, WI, CA). After washing three times for 40 min with 100 μL 50mM ammonium bicarbonate and 50% methanol, the gel pieces were dried and swollen in digestion buffer (20 mM ammonium bicarbonate and 40 ng/μL trypsin), and incubated at 37 °C for 16 h. Peptides were extracted twice by 20 min incubation in 35 μL of 50% ACN in 0.1% TFA. The resulting peptide extracts were pooled, concentrated in a vacuum centrifuge and stored at −20 °C.

6. Mass Spectrometry. Dried samples were dissolved in 10 μL of 30% ACN and 0.1% TFA. Equal volumes (0.5 μL) of peptide and matrix solution were mixed and crystallized on the sample plate. The matrix solution consisted of 3 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 mL of a solution containing 50% ACN and 0.1% TFA. Peptides were analyzed with an Applied Biosystems 4700 proteomics analyzer (Applied Biosystems, Framingham, MA) with TOF/TOF ion optics. MS spectra were obtained in reflectron mode using an acceleration voltage of 1 kV. Desorption and ionization was performed with an Nd:YAG laser operating at 355 nm, and the final mass spectrum was produced by averaging 3600 laser shots. The products of trypsin autodigestion were used for internal calibration. The following parameters were used for protein identification: 30 ppm error, optional oxidation of methionine and two possible missed cleave site. All MS/MS sequencing analysis were performed using the same equipment.

7. Protein Identification Using the *Drosophila* Database. The first step in protein identification consisted of mass fingerprinting. The mono-isotopic peptide mass fingerprinting data obtained from MALDI-TOF were then used to search for candidates in the SWISS-PROT/TrEMBL no redundant protein database.

8. Antibodies Against Mitochondrial Proteins. We generated an antibody against denaturated mitochondrial matrix P1 precursor polypeptide. The contents of 10 confluent 500 cm^2 trays (Nunc) of Schneider cells were used to prepare the polypeptide for immunization. The cell monolayer were washed with Hanks' solution, and scraped with a rubber policeman into 10 mL of the same medium. After centrifugation, the cells were sonicated, treated with DNase and RNase, and passed several times through a narrow-gauge needle. The sample was then lyophilised and resuspended in 5 mL of lysis buffer, and [^{35}S]methionine + [^{35}S]cysteine labeled proteins from wing imaginal disks were added to identify the polypeptides by autoradiography. One hundred 2-D gels were run as described above, immediately dried (without fixation) and exposed 4 days. The mitochondrial matrix protein P1 precursor was located by autoradiography and cut out of the gels. The gel pieces were rehydrated and the protein recovered by electroelution. Approximately 100 μg of this protein was injected into a New Zealand White rabbit, and its serum were tested by Western blotting, as described below.

To prepare antibody against native hsp60, a cDNA containing the complete open reading frame was cloned into the pRSET vector (Invitrogen, Leek, The Netherlands), and expressed as a His_6-fusion protein in *Escherichia coli* BL21 (DE3)pLysS (Novagen, Madison, WI). Approximately 240 μg of this protein

Table 2. Identification by MALDI-TOF and MALDI-TOF-TOF MS of Basic Polypeptides (NEPHGE) Recorded in the *Drosophila* Mitochondria Database

SSP no.	KDa/p*I*	N[a]/%[b]	sequence determined by MS/MS	protein identified	fly base ID FBgn
1202	13.1/9.82	6/47.0	933.55 LRQEFLK 1335.67 VLSNEEQEFIK 1406.70 VSNYEHFKPTGK 1558.75 HATGEGGTVFDAGLAR	CG 12859	0033961
1302	22.4/9.64	13/55.0	799.43 TIMAAHR 1101.61 DLTALQATIR 1582.88 VDPSIIGGLIVSIGDK 1741.93 FAPATVNLLGLLADNGR	OLIGOMYCIN SENSITIVITY CONFERRING PROTEIN Oscp	0016691
1408	24.1/9.35	16/52.0	923.48 WIHAPSGR 1067.55 SYILDGFPR 1127.61 NIAQAEALAAR 1641.82 VPGKDDVTGEPLMQR 2133.15 EQIDAVITLDVPHSVIIDR	ADENYLATE KINASE − 3	0042094
1508	34.6/9.81	16/39.5	1074.55 EEGLTALWR 1206.61 NYTNVANALAR 1505.72 LGMYTYLNDLFR 1641.91 EGPLALYQGIGAALLR	CG 1907	0039674
1509	33.0/9.55	16/67.9	956.59 QQVSLLIR 1057.53 QVAQQEAQR 1177.63 FNASQLITQR 1743.89 LGGIQSDIYSEGLHVR 2376.21 VLAAVGAAAYGVSQSLYTVEGGHR	l (2) 03709	0010551
2508	35.3/9.20	10/37.2	1205.68 LFGVSTLDVVR 1265.71 GSDVVVIPAGVPR 1318.69 DDLFNVNAGIIK 1492.75 TAGFIGADQLGDSLK	CG 7998	0038587
2608	45.4/9.47	20/71.6	1177.59 NGLGNSIYSPR 1238.55 AASANWYGGDAR 1602.77 NESYDIQGASHLLR 1645.82 NIQQVGGTLTTWGDR 2388.25 YLQDLLQPAFKPWELVDNAK 2540.34 NVLEADALLGAIDGISQSQVQEAAK	CG 4169	0036642
2613	47.1/8.95	21/50.7	934.51 INLGAGAYR 1267.64 GLYSNPPVHGAR 1487.73 DDNTQPFVLPSVR 1511.78 HNVTAQSISGTGALR 2510.24 STWFSEVQMGPPDAILGVTEAFK	GLUTAMATE OXALOACETATE TRANSAMINASE 2 Got 2	0001125
2810	79.6/8.96	30/46.8	789.50 GLIALFR 1040.54 GIFLYDGQK 1233.68 ILSTMLSEAIR 1581.82 VVITVGDGPGFYTTR 1836.02 LGLPEVMLGLLPGGGGTVR 1969.98 DQTLASLRPTLDYSDFK	CG 4389	0028479
3413	26.5/7.69	17/64.2	1135.59 NGFLLDGFPR 1204.56 SYHEEFAPPK 1452.78 QTKPLVDYYGLR 1601.77 SSDVFSTIDSIFQR 2004.05 TNLDAVIEFAIDDSLLVR	ADENYLATE KINASE − 2 Adk 2	0022708
3708	81.9/8.99	31/39.0	1019.51 GVYPEDALR 1122.57 LDTYDILNR 1358.66 AQLEYHLEAER 1412.76 GDDLVAAVLESVPK 1561.78 AVENGENSLWTTLK	CG 6455	0019960
3714	60.9/8.44	23/45.9	1059.53 NLNHVSYGR 1389.60 DAGNYEMITGYR 1537.68 FFDMVEYFFHR 1746.91 TFIVQGFGNVGLHTTR 1932.91 ESNYHLLESVQESLER	GLUTAMATE DEHYDROGENASE Gdh	0001098
3813	85.3/8.49	25/35.0	1102.61 LQLLEPFDK 1215.59 NGSWGGVPDVAR 1457.77 IPFNVTPGSEQIR 1625.72 WVAVGDENYGEGSSR 1698.90 LNHTLNDLQIGWFK 1854.94 VLYSHLDDPANQDIVR 2076.94 GFDPGQDTYTAPPPSGENVK	ACONITASE Acon	0010100
4308	18.9/6.52	7/45.9	1040.48 FEADQQFR 1128.53 YGDLGGYANAK 1315.70 MVDNEIVNILR	Pdsw	0021967
4310	24.8/8.88	13/42.6	2527.36 KGPAPLNLEVPTHEFPNEG LLVVG	RIESKE IRON-SULFUR PROTEIN RFeSP	0021906
4409	27.2/7.76	14/61.4	1352.73 IEVISVEDPPVR 2095.12 LAEEVIAVSVGPAQSQEVIR	CG 7834	0039697

Table 2. (Continued)

SSP no.	KDa/p*I*	N^a/%^{b}	sequence determined by MS/MS	protein identified	fly base ID FBgn
4504	31.0/7.69	10/44.8	1216.58 DFSVVYDLMK 1329.66 DYAGGFSSALITK 1750.90 DTIFIDSSTISPDLVK 1891.93 QGLDANVFAEIINSSTGR 2289.22 DLGLASGVANASNSPIPLGSLAHK	CG 15093	0034390
4609	50.4/7.15	25/63.3	989.47 YYGAQTMR 1124.57 VESDTFGELK 1389.74 SQINFPIGGATER 1791.99 VYELALGGTAVGTGLNTR 2027.10 DAMVEVHGVLNTIAVSLMK	1(1) G0255	0028336
4703	61.0/8.58	20/37.7	1746.91 TFIVQGFGNVGLHTTR	GLUTAMATE DEHYDROGENASE Gdh	0001098
4721	59.3/9.09	17/35.0	1026.58 AVDSLVPIGR 1438.84 GIRPAINVGLSVSR 2365.14 EVAAFAQFGSDLDAATQQLLNR	BELLWETHER (blw) ATP SYNTHASE ALPHA	0011211
4802	80.4/7.56	21/29.3	855.48 WPIIGNR 1205.68 VIFFLPWQR 1290.69 AILGLDLEQYR 1599.80 IHPEFPYIDAEIR 1858.86 SFGDGDVSGEQLHEILR	CG 8256 1(2) K05713	0022160
5401	30.5/6.44	20/86.0	1505.77 VNNASQVGLGYQQK	PORIN	0004363

^{a} *N* = Number of matched peptides. ^{b} % = Coverage of full length protein by tryptic peptides.

were purified by NiTA chromatography (Clontech, Palo Alto, CA) and injected into a New Zealand White rabbit. The rabbit serum was tested by immunostaining as described below.

9. Immunobloting. Proteins resolved by 1-D gels were transferred onto nitrocellulose filters at 130 mA for 6 h, and the filters incubated for 120 min at room temperature in PBS containing 10% bovine serum and α-mitochondrial matrix precursor diluted to 1:100. They were then incubated with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) for 120 min in the same buffer and immunocomplexes visualized with diaminobenzidine as substrate.

10. Immunostaining of Imaginal Disks. Standard procedures were used for whole-mount immunohistochemistry.[14] Imaginal disks were stained as described.[15] The α-Hsp60 was used at a 1:2000 dilution in all experiments. Topro was also used to stain nuclei, and Alexa Fluor-488 (Molecular Probes) to detect the primary antibody.

11. Microscopy and Image Treatment. Imaginal disks were mounted in Vectashield (Vector Laboratories, Inc). Images were acquired with a BioRad 2000 confocal microscope and treated with Metaview (Universal Imaging) and Photoshop 7.0 (Adobe Corp.) software. We captured at least 45 planes of the imaginal disks, and later merged planes corresponding to different surfaces of the imaginal disks.

Results

Isolation of Mitochondrial from *Drosophila* Third Instar Larvae. Figure 1 summarizes the method used to purify mitochondria from third instar larvae of *Drosophila melanogaster*. We obtained a yield of 5 mg of mitochondrial protein after homogenizing 5000 third instar larvae. The progress of purification was followed Western blotting with a polyclonal antibody against the mitochondrial matrix protein P1 precursor (Figure 1B). We identified this polypeptide some years ago as IEF 16 in our manual database[16] and have generated an antibody against the denatured peptide (see Materials and Methods). The intensity of the Western bands was 40 times greater in the purified mitochondria than in the initial homogenate. The functional integrity of the purified mitochondria was evaluated by assaying citrate synthase and the individual complexes of the respiratory chain. Specific activities in the final preparation were always at least 50 times higher than in the initial homogenate. Finally, the structural integrity of the purified mitochondria was confirmed by electron microscopy. Figure 1C shows a small but representative field in which one can see that the majority of mitochondria are intact.

General Pattern of Mitochondrial Protein of *Drosophila.* Four hundred micrograms of mitochondrial protein obtained as described above was lyophilized, resuspended in lysis buffer and analyzed by 2D gel electrophoresis. Figure 2 shows a representative 2-D gel of the silver stained acidic polypeptides of the mitochondria of mature larvae. Gels were analyzed with the PDQUEST system, and each polypeptide was assigned a number in the database. In this way we compiled a database of 231 silver stained polypeptides (143 acidic, IEF and 88 basic, NEPHGE). This constitutes our reference mitochondria database.

Isolation of Proteins for MALDI-TOF. After calibration with Coomassie blue-stained 2-D gels, we ran preparative gels with precise amounts of purified mitochondria from third instar larvae. After staining we picked some of these spots and subjected them to in-gel digestion with trypsin and MALDI-TOF analysis. By this approach we were able to identify 66 spots (44 acidic and 22 basic) in the preparative gels of the database. In all cases analyzed, we were able to confirm the information obtained from the peptide mass fingerprinting by tandem mass spectrometry. A total of 248 MS/MS spectra were analyzed. All the mitochondrial proteins identified in this study are listed in Tables 1 and 2, ordered by SSP number. Molecular weight, isoelectric points, names, identified sequence tags and Flybase accession numbers are also included. As an example, Figure 3A shows, the MALDI-TOF spectrum of the tryptic digest of basic polypeptide SSP 3813 identified as aconitase, and Figure 3B reproduces a detail of the fragmentation spectrum of peak 1457.77 corresponding to the polypeptide IPFNVTPGSEQIR.

Mitochondrial Pattern in Imaginal Disks. We generated an antibody against a native mitochondrial protein in order to use it as a cytological marker. Heat shock protein 60 (SSP 8705 in the database) was selected for that purpose and Figure 4 illustrates the specificity of the antibody using the third instar

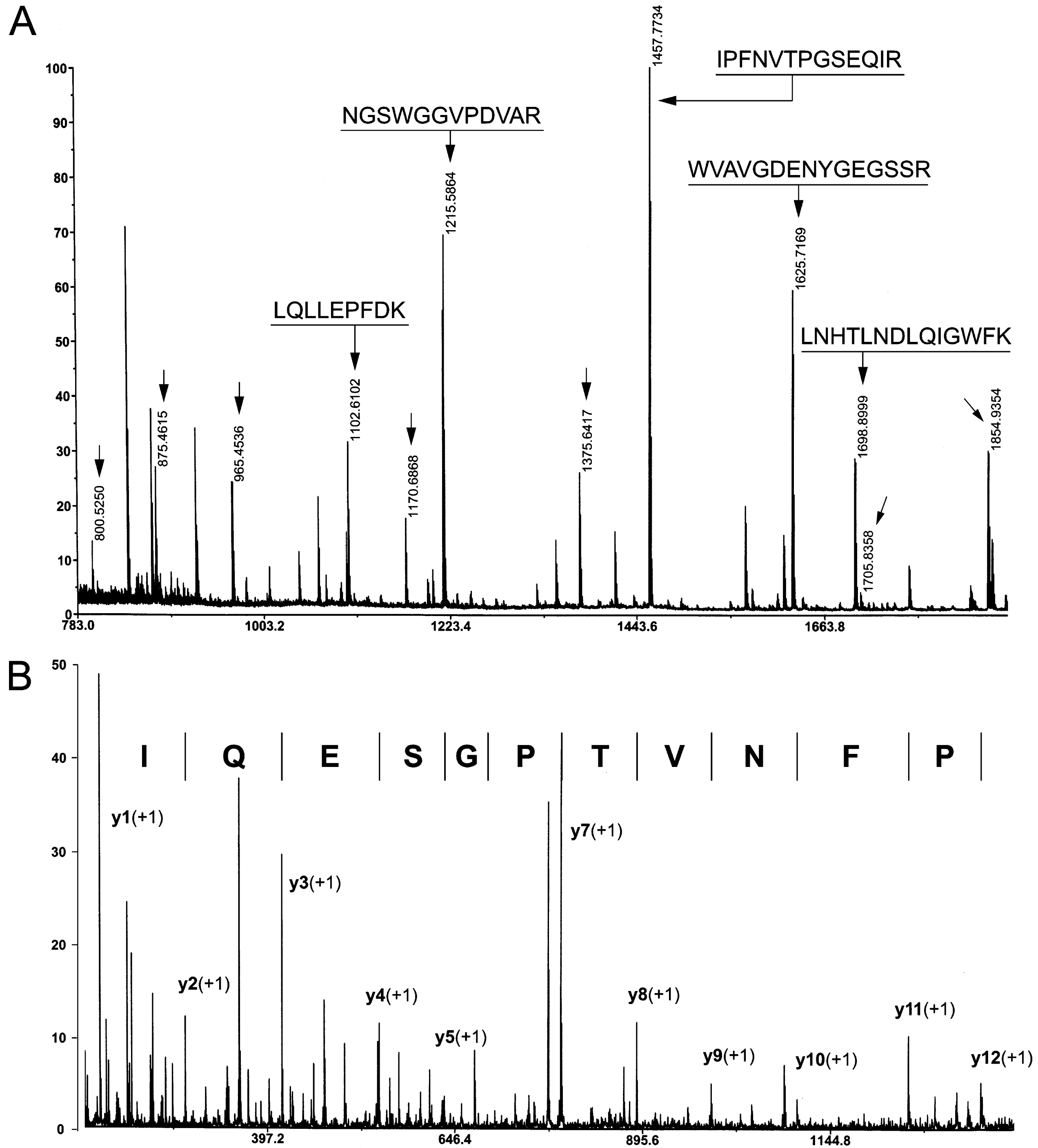

Figure 3. (A) MALDI-TOF MS spectra of the tryptic digest of basic polypeptide SSP 3813. The labeled peaks represent 12 of the 25 peptides matched to *Drosophila melanogaster* aconitase. Some of the peptides analyzed by MALDI-TOF/TOF MS are also indicated with its sequences underlined. (B) A detail of the MS/MS spectrum of peptide 1457.71 Da, matched to the sequence IPFNVTPGSEQIR.

wing imaginal disks, the structure generally used by us as a model. Topro (blue channel) was used to stain nuclei, and it is possible to see well-defined zones of mitochondrial staining (green channel) surrounding the nuclei, both in the epithelial cells (Figure 4A) and in the peripodial epithelium (Figure 4B). The diameters of these cells are highly variable, ranging from the large flat cells of the squamous peripodial epithelium (20–30 μm in diameter) (Figure 4D) to the tall, narrow cells of the columnar epithelium (2–6 μm in diameter) (Figure 4C). Figure 4E,F shows the transverse views of the two epithelial layers making up the disk. The mitochondria are clearly concentrated toward the apical surface of these cells. Our antibody stained the mitochondria of the salivary glands, central nervous system and all the imaginal disks of third instar larvae and pupae (data not shown).

Discussion

The aim of this study was to create a protein map of the mitochondria of *D. melanogaster* that can serve as reference database for further studies. The present experimental approach has enabled us to identify 66 spots by peptide mass fingerprinting, and to identify them from their MS/MS spectra

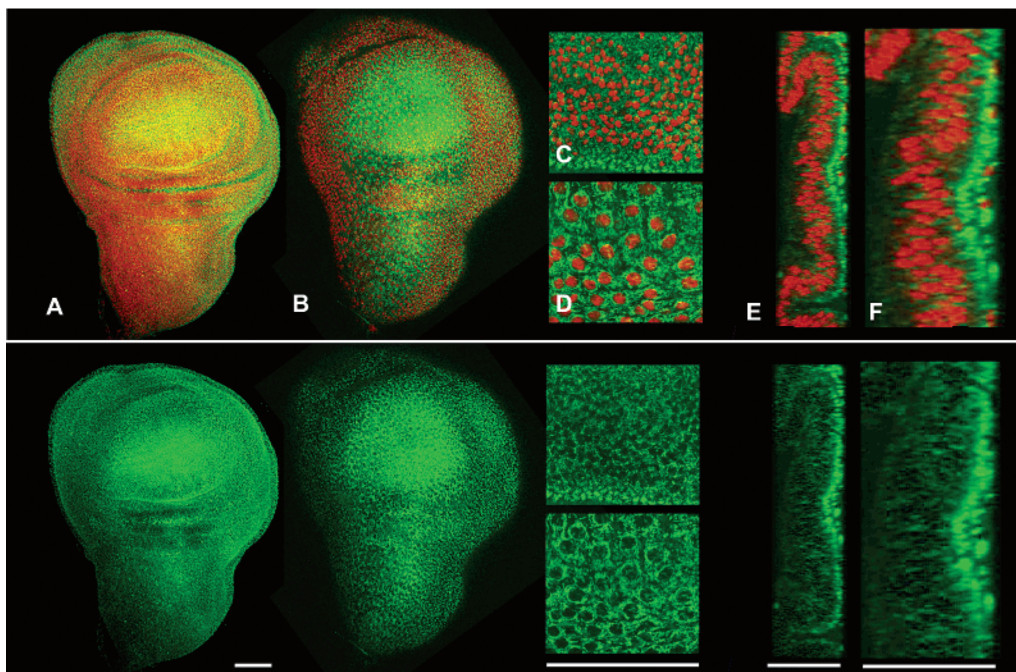

Figure 4. Expression of mitochondrial hsp60 in third instar *Drosophila melanogaster* wing imaginal disks. A, C, and E: general (A), detailed (C) and transverse (E) views of epithelial cells. B, D, and F: general (B), detailed (D) and transverse (F) views of the peripodial epithelium. In all cases expression of hsp60 is shown in the green channel while topro is shown in the blue channel. White bar = 100 μm.

in the *Drosophila* mitochondrial proteome. Tables 1 and 2 list the polypeptides identified in the database and include the corresponding accession numbers in the *Drosophila* genome database (http//flybase.bio.indiana.edu), which is possibly the most complete currently available database of any eukaryotic organism, with extensive genetic and molecular information about each polypeptide.

The 66 identified spots represent 28.5% of the 231 polypeptides recorded in the database, but in some cases the same protein was found in several spots. Such multiple spots could stem from highly similar isoforms or, most probably, post-translational modifications. Five instances where two spots contained the same polypeptide were detected among the acidic gels: heat shock protein 60 (hsp60), l(1)G0030, l(1)G0334, CG 3902, and CG 3731. Another five instances where two spots contained the same polypeptide were detected on the acidic and basic gels: aconitase, Rieske iron−sulfur protein, Pdsw, adenylate kinase-2 and CG 15093. Finally, we detected five instances where three spots contained the same polypeptide: ATP synthase beta chain, CG 3752, Porin, l(1)G0255 and glutamate dehydrogenase. In summary, this means that the identified spots actually represent 46 different protein species. We detected only one protein fragment, corresponding to acidic polypeptide SSP 9405, a fragment of the ATP synthase beta fragment. The other 65 identified spots were full-length polypeptides.

Mitochondria contain their own DNA (mtDNA), a covalently closed duplex of about 19 500 base pairs. *Drosophila* mtDNA encodes thirteen hydrophobic polypeptides, which have been identified as subunits of enzyme complexes associated with the inner mitochondrial membrane, a large and small ribosomal RNA and 22 transfer RNAs.[17] The nuclear genes encoding the polypeptides identified in this study mapped over the four *Drosophila* chromosomes in proportions consistent with the sizes of the latter: 20 polypeptides on chromosome 2, 17 on chromosome 3, 7 on the X chromosome and 2 on chromosome 4.

All mitochondrial locations are represented by the identified polypeptides. The mitochondrial inner membrane is most highly represented with 22 polypeptides, followed by mitochondrial matrix with 21 polypeptides, the mitochondrial outer membrane with two polypeptides and the intermembrane space with one. These data thus validate our methods of sample preparation and extraction.

In most of the analyzed cases, experimental M_r values were in good agreement with the theoretical values. In contrast, the apparent p*I* values of the proteins tended to be more acidic than predicted, suggesting that the detected polypeptides were highly phosphorylated.

The proteins identified cover a wide spectrum of biological functions. For example, 17 are involved in oxidative phosphorilation (7 in Complex I: NADH ubiquinone oxidoreductase, 4 in Complex III: ubiquinol-cytochrome C oxidoreductase, 1 in Complex IV: citochrome c oxidase and 5 in Complex V: F0/F1 ATP synthase). Nine are involved in carbohydrate metabolism (6 in the tricarboxylic acid pathway and 3 in pyruvate metabolism). Five are involved in amino acid metabolism and four in the metabolism of complex lipids (one in glicerolipid metabolism and three in fatty acid metabolism). Two are involved in nucleotide metabolism and one in DNA metabolism. Three are involved in protein manipulations (one in protein folding and stabilization, one in protein translocation and one in proteolysis). Two are involved in transport and one in cell defense. Finally, one is involved in larval development and another, NEPHGE SSP 3708, is of unknown function. The major benefit of these database is to provide a comprehensive resource for the discovery of novel mitochondrial functions and pathways in *Drosophila*. The database could also be used in

future to analyze the protein alterations associated with mitochondrial diseases. The antibody against native mitochondrial heat shock protein p60 generated in this study, may be a useful complementary tool in such work. For example, it revealed a specific apical localization for the organelles in cells of the wing imaginal disk (Figure 4).

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