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BIOCHEMICAL CONSEQUENCES OF A LARGE DELETION IN THE MITOCHONDRIAL GENOME OF A *DROSOPHILA SUBOBSCURA* STRAIN

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A mutant strain of *D.Subobscura* possesses two populations of mitochondrial genomes : a population identical to that of the wild strain (20%) and a dominant population (80%) which has lost more than 30% of its coding zone by deletion. Spectrophotometric determination of respiratory complex activities shows that : complex I (5 genes implicated in deletion) presents maximal activity reduced by 40%, whereas that of complex III (concerned by cytochrome b) is lowered by 30%. Nevertheless, polarographic determinations of substrate oxidation show activity of complex I to be reduced by 30%. In contrast, complex III activity is similar to that measured in the wild strain. The predominant use of one part of the respiratory chain may account for the fact that the mutant strain is apparently unaffected by mutation.

A mutant strain of *D. Subobscura* possesses two populations of mitochondrial genomes : a minority population (20%) with coding capacity identical to that of the wild population, and a dominant population (75-80 %) which has lost by deletion more than 30% of the coding zone (1). Deletion affects five genes from complex 1 (5'end of ND1, ND6, ND4, ND4L; 3' end of ND5), the cytochrome b gene, and 4 tRNA (Ser, Pro, Threo, His). Heteroplasmy to this extent means that only 20% of the mitochondrial genomes have stored this information for subunits of the concerned I or III complexes. Deletions, thus represented, cause severe pathologies in man, which

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can be correlated with dysfunction of the mitochondrial respiratory chain (2-4). The mutant strain does not seem to be significantly affected (1). The study of mitochondrial genome expression (5) has shown that : a) deleted and intact genomes are expressed ; b) transcripts of genes, unaffected by mutation, are present in identical concentrations in the wild and mutant strains, whereas transcripts of affected genes account for a lower concentration in the mutant strain, despite a significant increase (50%) of the mtDNA present in the cells. In this study, we analyse the consequences of deletion on the activity of various respiratory complexes, and on mitochondrial respiratory capacity.

METHODS

Mitochondria were isolated from a ground preparation of adult flies of wild or mutant strains in keeping with the previously described method (6). Protein levels were determined by Bradford's method (8). The isolated mitochondria were sonicated for 6 seconds for spectrophotometric determination of respiratory complex activities. Activity of complex I (NADH-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome C reductase), and complex IV (cytochrome oxidase) was determined by respectively applying the methods previously described (9, 10, 11).

Mitochondrial respiration was measured by polarography in a 29°C thermostatically controlled cell (Hansateck cell). For each test, 0.3-0.5 mg of mitochondrial protein was incubated in 1 ml of medium consisting of : 0.15 M sucrose, 0.1 M mannitol, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM KH_2 PO_4, 0.5 mM ADP, and 20 mM pH 7.5 Tricine. After 2 minutes of incubation, the substrates were added as small aliquots (5 µl). The quantity of consumed oxygen was calculated as described by Estabrook (12).

RESULTS AND DISCUSSION

1 - Spectrophotometric measurement of activities

Results of activity measurements of complex IV, unaffected by deletion, and complexes I and III, affected by deletion, are presented in Figure 1. Activity of complex IV is identical in both strains, respectively 2519 ± 992 nmoles of reduced cytochrome C/min/mg in the wild strain (W), and 2551 ± 1053 in the heteroplasmic strain (H). Activity of complex I (W = 467 ±(89) nmoles/min/mg; H = 279 (±65))is lowered in the heteroplasmic strain (40 ± 7%). Activity of complex III (W = 588 (± 200) nmoles/min/mg;

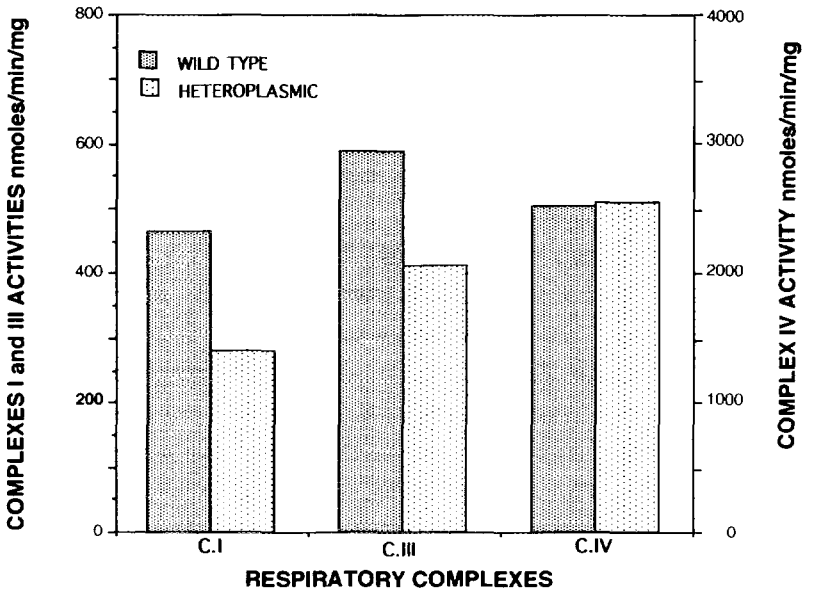

Figure 1 . Respiratory complex activities

Activity of the enzymatic complexes of the respiratory chain of the wild strain (W) and the heteroplasmic strain (H) were measured by spectrophotometry. Complex I was measured in the presence of ubiquinone 100 µM , complex III in the presence of cytochrome C 15 µM and ubiquinol 15 µM, and complex IV in the presence of reduced cytochrome C 35 µM.

H = 414 ± 174)) is also lowered, but to a lesser extent (30 ± 13%). Statistical studies (Student tests) indicate that the decrease of the activities are highly significant for the complex I (16 experiments), significant for the complex III (13 experiments). No difference is statistically observed for the complex IV (16 experiments).Complex IV shows identical activity in both strains, so the Complex I/Complex IV and Complex III/Complex IV ratios are lower in the heteroplasmic strain.

Deletion has a clear incidence on activity of the two complexes concerned by mutation. In both cases, it is less than the level of heteroplasmy (70 to 80% in favour of the deleted genomes), as it was already observed for relative concentrations of mitochondrial transcripts in steady state (5). Nevertheless, this influence is not equivalent for the two complexes. For complex III, the decrease in activity is equivalent to that measured for the cytochrome b transcript concentration in the mutant strain compared with the wild strain (34%). This decrease in activity may thus be directly related to the decrease in cytochrome b

concentration, hence a decrease in functional complex III concentrations in the membrane.

For complex I, the decrease is similar to that measured for the single transcript of the ND4 and ND4L subunits (45%). It is less than the decrease in transcript levels for subunits ND1 and ND5 (respectively 55 and 65%).

Several hypotheses may account for these results. For the ND5 and ND1 transcripts, partial compensation may occur at the translation level (overtranslation of transcripts). Another explanation may be the fact that lowered concentration of these two latter polypeptides exerts only a limited influence on complex activity. The exact function of mitochondrial subunits has yet to be clearly defined. The ND1 subunit may play a role in rotenone and ubiquinone binding (13,14). The ND4 subunit is apparently implicated in electron transfer from the mitochondrial matrix to the respiratory chain (14). This subunit is thus determinant as regards the relationship between matricial dehydrogenases (via NADH) and complex I. This could account for the parallel between the decrease in activity measured in this complex, and the ND4 transcript level.

2 - Measurement of substrate oxidation capacity

Mitochondrial respiratory capacity was tested by polarography in the presence of various substrates (Table 1). Results obtained with *D. Melanogaster* are presented for comparison ; this strain was the subject of a previous study (6,7). Oxygen reduction capacity without any additions (endogenous respiration) was very similar in the three strains. Nevertheless, it was slightly higher for the mutant strain (16%), even in the presence of rotenone. This may indicate that these endogenous substrates are complex III substrates. Oxygen reduction kinetics in the presence of glutamate-malate (electron donors to complex I via NADH) were similar in *D. Melanogaster* and the *D. Subobscura* wild strain. They were lowered in mitochondria of the heteroplasmic strain (30%). The addition of succinate, allowed complex III function to be tested. Both *D. Subobscura* strains showed identical oxidative capacity, 10% lower than that of *D. Melanogaster*. This oxidative capacity is however low, even in the presence of a high substrate concentration (5 mM). The addition of α-glycerophosphate, known to be a good substrate for insect muscle mitochondria (15,16) strongly stimulated oxygen reduction. For a 4 mM α-glycerophosphate concentration, oxygen

Table 1 . Oxygen reduction by mitochondria incubated with different substrates

STRAINS	SUBSTRATE			
	no addition (endogenous respiration)	Glutamate + Malate	Succinate + rotenone	α-Glycero. + rotenone
D. Melano.	29.8 +/- 5 (13)	69.2 +/-13 (8)	97 +/- 15 (6)	_
D. Subob. W	33.8 +/- 8 (25)	60.5 +/- 16 (12)	88 +/- 19 (5)	248 +/- 123 (8)
D. Subob. H	39.4 +/- 9 (10)	44.5 +/- 12 (8)	87.4 +/- 6 (5)	240 +/-106 (8)

Mitochondria (0.3 mg) isolated from *D. Melanogaster*, wild type (W),or mutant (H) *D. Subobscura* strains were incubated at 29°C. without (no addition) or with different substrates as indicated in Methods. Glutamate and malate were 5 mM. Succinate and α- glycerophosphate were 5 mM. Rotenone (1µg/ mg protein) was added for these two last incubations. Experiment numbers are indicated in the parentheses. Results were expressed in natoms/min/mg.

reduction kinetic was 2.8 times greater than that measured with the succinate. Figure 2 shows a direct relation between the oxidation rate of α-glycerophosphate and its concentration. There are no statistical differences between the two strains. The oxidation of this substrate allows partial respiratory chain function; indeed, α-glycerophosphate-oxidase is located on the outer surface of the internal membrane (17).This flavoprotein gives up its electrons to complex III via ubiquinone. Thus, contrary to succinate, there are no problems related to substrate penetration in the mitochondrial matrix.

Polarographic results clearly differ from spectrophotometric results. With substrates which reduce complex I, the mutant strain presents reduced respiratory activity, albeit to a lesser extent (30% compared with 40%). But with complex III substrates, there is no longer a detectable difference. Although this method constitutes a more physiological approach to the respiratory chain, it does not allow evaluation of maximal electron transfer capacities for the various respiratory complexes : for example, for the wild strain,

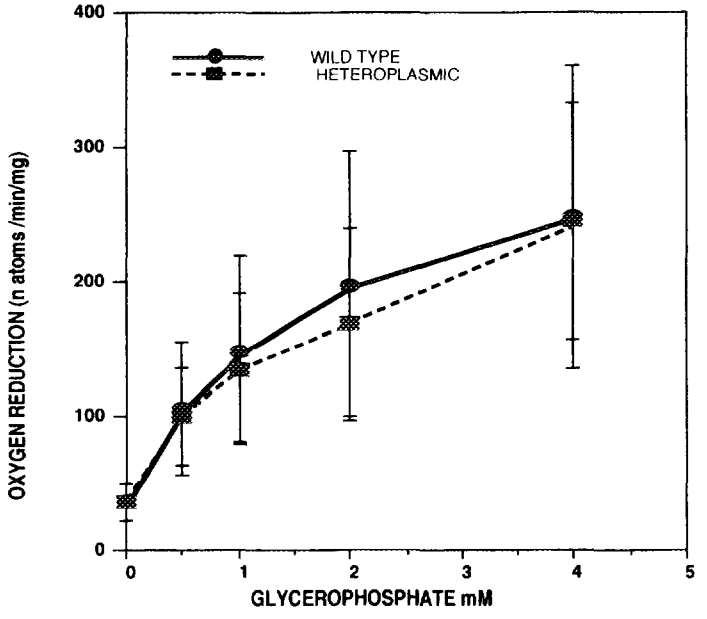

Figure 2 . Oxygen reduction by isolated mitochondria incubated with α-glycerophosphate

Mitochondria isolated from the wild strain (W) or the mutant strain (H) flies were incubated with various concentration of α-glycerophosphate in presence of ADP 0.5 mM and rotenone 1 µg/mg protein. Oxygen reduction was measured as indicated in "Methods".

with specific complex I substrates, reduction of 60.5 natoms of Oxygen/min/mg corresponds to oxidation of 60.5 nmoles of NAD. Much higher activity is measured with spectrophotometric method (467 nmoles/min/mg). Similarly, with succinate or with α-glycerophosphate, oxygen reduction kinetics correspond to complex III activity which substantially differs from that measured spectrophotometrically. *In situ* functioning of the respiratory complexes is therefore consistently below potential : only a fraction of activity is used. Nevertheless, consequences of deletion on complex I are always detectable, despite the fact that they are less marked, even well within maximal activity. One or several of the subunits concerned by mutation is or are thus indispensable for proper functioning of the complex (even at a non-maximum rate). Moreover, it should be pointed out that this complex presents by comparison with complex III or IV, the lowest activity (Figure 1). It may represent the master reaction in respiratory chain function. For complex III, these differences are no longer detectable by determination of oxygen reduction.In this

case, "residual" activity of complex III (70% of the activity of the wild strain), may be sufficient, under our measurement conditions (and possibly *in vivo*), to ensure normal electron transit despite the intrinsic alteration observed in maximal activity. Futhermore, potential activity of this complex is higher than that of complex I (see Figure 1).

CONCLUSION

In human pathology, even if it is not always possible to correlate deletion with direct respiratory chain effects (18), enzymatic complex dysfunctions are frequently encountered and have severe consequences. For the mutant strain, these alterations apparently have no direct effect on fertility rates, larval or imago emergence (1). The fly must thus have developed compensatory mechanisms to maintain cellular energetic equilibrium. In particular, such mechanisms ensure efficient exploitation of the part of the respiratory chain from complex III which, under *in situ* conditions, shows practically normal functioning. Studies of these metabolic pathways are currently underway in our laboratory.

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REFERENCES

1 - Volz-Lingenhol A., Solignac M., Spierlich D.A. (1992) Proc. Natl. Acad. Sci. USA 89, 11528-11532.
2 - Holt I.J., Harding A.E. and Morgan-Hughes J.A. (1988) Nature 331, 717-719.
3 - Holt I.J., Harding A.E., Cooper J.M., Shapira A.V.H., Toscano H., Clark J.B. and Morgan-Hughes J.A. (1989) Ann. Neurol. 26, 699-708.
4 - Zeviani M., Moraes C.T., Di Mauro S., Nakase H., Bonilla E., Schon E.A. and Rowland L.P. (1988) Neurology 38, 1339-1346.
5 - Beziat F., Morel F., Volz-Lingenhol A., Saint-Paul N. and Alziari S. (1993) Nucl. Acids Res. 21, 387-392.
6 - Alziari S., Stepien G.and Durand R. (1981) Biochem. Biophys. Res. Commun. 99, 1-8.
7 - Alziari S., Berthier F., Touraille S., Stepien G. and Durand R. (1985) Biochimie 67, 1023-1034.
8 - Bradford M. (1976) Anal. Biochem. 72, 248-251.
9 - Hatefi Y. (1978) Methods Enzymol. 53, 11-14.
10 - Hatefi Y. (1978) Methods Enzymol. 53, 35-40.
11 - Appelmans F., Wattieux R. and De Duve G. (1955) Biochem. J. 59, 438-445.
12 - Estabrook R.W. (1967) Methods Enzymol. 10, 41-49.
13 - Ragan I. (1987) Current Topics in Bioenergetics 15, 1-37.

14 - Majander A., Huoponen K., Savontaus M.L., Nikoskelainen E. and Wikström M. (1991) Febs Letters 292, 289-292.
15 - Estabrook R.W. and Sacktor B. (1958) J. Biol. Chem. 233, 1014-1019.
16 - Sacktor B. (1974) In The Physiology of insects (Rockstein M. ed.) 4, pp. 271-354. Academic Press, New York.
17 - Klingenberg (1970) Eur. J. Biochem. 13, 247-252.
18 - Osawa T., Yoneda M., Tanaka M., Ohno K., Sato W., Suzuki H., Nishikimi M., Yamamoto N., Vonaka I. and Horais (1988) Biochem. Biophys. Res. Comm. 154, 1240-1247.