ELECTROPHORETIC PATTERN OF AND AMINO ACID INCORPORATION *IN VITRO* INTO THE INSOLUBLE MITOCHONDRIAL PROTEIN OF NEUROSPORA CRASSA WILD TYPE AND MI-1 MUTANT

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1. Introduction

The maternally inherited (mi) mutants of *Neurospora crassa* exhibit polyphenic deviation in mitochondrial composition and function [1]. For instance in the first days of mycelial growth, the concentrations of cytochrome a and b are drastically reduced and those of cytochrome c and levels of fatty acids increased in the mutant compared to the wild type [2,3]. In this stage respiration is virtually cyanide insensitive [4]. These effects may originate from alterations of the amino acid sequence of 'structural protein', which is regarded as important in the organisation of the electron transport chain [5]. The mitochondrial DNA was thought to contain information for the amino acid sequence for structural protein and the mitochondrial deviations would originate from point mutations. However preparations of 'structural protein' have been resolved electrophoretically into several fractions [6, 7] and the experimental basis of the explanation of Woodward et al. is open to criticism. Recently techniques for the electrophoretic fractionation of complex insoluble mitochondrial proteins after incorporation of labelled amino acids *in vitro* have been developed [6, 8]. In the experiments described here these techniques have been applied to the insoluble protein fraction of mitochondria (IMP) from the wild type and from mi-1.

2. Methods

Strains and growth conditions. The mutant mi-1 of *N. crassa* (3637-1A) was a generous gift of M. B. Mitchell. Hyphae were grown in Vogels minimal medium [9] plus 2% glucose with shaking. The inoculum was 1.5×10^6 conidia per ml for wild type and 10^5 for mi-1. The wild type was harvested after 24 hours and the mi-1 mutant after 72 hours. Mitochondria were prepared after enzymatic degradation of the cell walls according to Greenawalt et al. [10].

Preparation of ^{14}C-labelled IMP: Isolated mitochondria were incubated at pH 7.6 for 30 min at 30° in a medium containing: sucrose 100 mM, KCl 50 mM, triethanolamine 30 mM, EDTA 2 mM, $MgCl_2$ 10 mM, K_2HPO_4 15-30 mM, bovine serum albumin 1.5 mg per ml, amino acid mixture 0.3 mg per ml (free of leucine, isoleucine and phenylalanine), mitochondria about 1 mg per ml and 1 μC per ml ^{14}C-*l*-leucine (u. 251 mC/mMole), 1 μC per ml ^{14}C-*l*-isoleucine (u. 262 mC/mMole) and 1 μC per ml ^{14}C-*l*-phenylalanine (u. 366 mC/mMole). Mitochondria of wild type readily incorporated amino acids when supplemented with ADP (2 mM) and α-ketoglutarate (5 mM) or ATP (4 mM). Mitochondria of the mi-1 mutant however required the presence of ATP (4 mM). After incubation, the mitochondria were washed three times with sucrose medium (sucrose 0.25 M, triethanolamine 10 mM and EDTA 2 mM), containing unlabelled leucine, isoleucine and phenylalanine. To remove soluble protein the mitochondria were extracted 3 times with 0.1 M phosphate buffer after sonifying 4 times for 15 sec. Lipids were extracted with acetone, chloroform/methanol (2 : 1) and once more with acetone. Electrophoresis was performed in a 7.5% polyacryl-

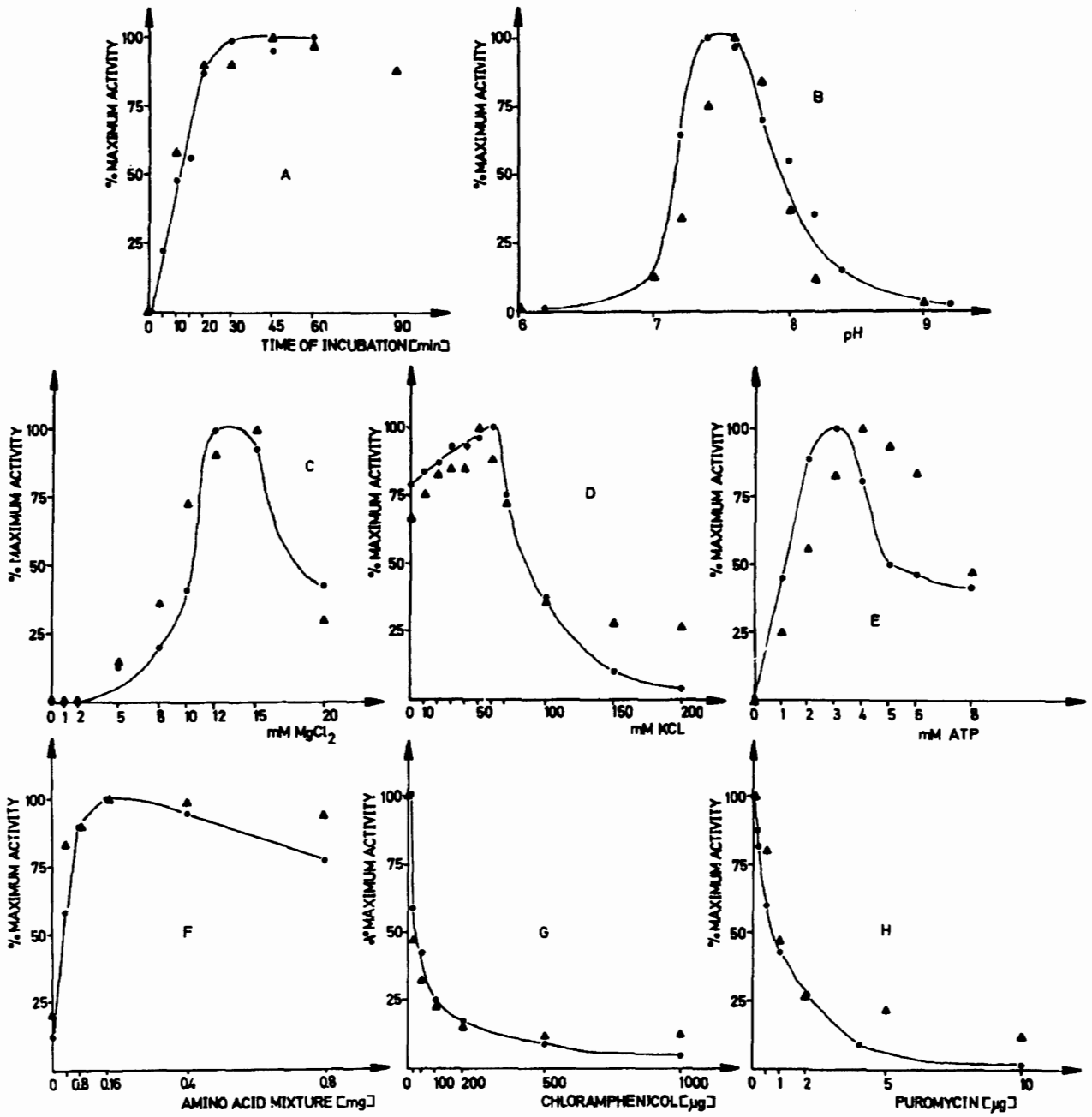

Fig. 1. Incorporation of amino acids into protein of isolated mitochondria as a function of incubation time (A), pH (B) and the concentrations of $MgCl_2$ (C), KCl (D), ATP (E), amino acid mixture (F), chloramphenicol (G) and puromycin (H) in the incubation medium. Circles and lines represent experiments with wild type triangles with mi-1 mitochondria. The complete incubation medium is that described under methods with the exception that only ^{14}C-*l*-leucine was used as precursor.

amide gel, equilibrated with a medium containing phenol, formic acid and water (2 : 1 : 1 w/v/v) [11]. The protein was dissolved in the same medium at a concentration of 10 mg per ml. The amido black-stained protein bands were measured at 546 mμ in a densitometer. For radioautography the stained gel was dried between a filter paper and a plastic sheet under an infrared lamp and exposed to an Osray D-W X-ray film.

3. Results

The incorporation rate was found to be about 10 times higher in wild type than in mi-1 mitochondria. Under optimal conditions wild type mitochondria incorporated about 300 pMoles leucine per hour per mg protein. No significant differences in the incorporation conditions for both types of mitochondria were apparent (fig. 1, A-F). Inhibitors of protein synthesis were equally effective in both types of mitochondria (fig. 1, G, H). Actinomycin C and D as well as cyclohexamide did not inhibit.

As described for other types of mitochondria [12, 13] the incorporated activity appeared mainly in that mitochondrial fraction which is insoluble after sonifying in 0.1 M phosphate buffer-95% in wild type and 90% in mi-1 and only these insoluble mitochondrial proteins (IMP) were used in our electrophoretic studies. The phenol medium used in the electrophoresis dissolves the mitochondrial proteins almost completely (fig. 3b). Protein and radioactivity remaining at the origin are less than 5% of the total. 20 bands can be observed. In fig. 2 these are numbered beginning with the slowly moving bands. Subbands resulting from improved separation are marked by letters. The IMP amounts to about 50% in both wild type and mi-1. The electrophoretic patterns of the IMP's isolated from both types are analogous. Bands 6a and 6b form the most prominent peak of both patterns. They are found in the same percentage of the total in both IMP's. Bands 10 and 11 are the major bands of 'structural protein' isolated according to Criddle et al. [14] from wild type mitochondria (Sebald, unpublished data). We find a higher fraction of the total in band 10 in the wild type IMP whereas band 11 is comparable. Band 4, containing a considerable fraction of the incorporated radioactivity, is nearly absent in mutant IMP. Differences are also observed in the fast moving bands. Resolution of the pattern is incomplete in both pherogramms in spite of the sharpness of single peaks suggesting a more complex protein pattern than shown in the pherogramm. Radioactivity incorporated *in vitro* shows peaks coinciding with protein bands both in the wild type and in the mi-1 pherogramm. In wild type IMP, bands 4, 6a and 7 plus 8 (containing 13, 8 and 12% respectively of the total radioactivity) are heavily labelled. In addition smaller amounts of radioactivity are found in bands 1, 2, 3, 13, 15 and 19 (fig. 2,3b).

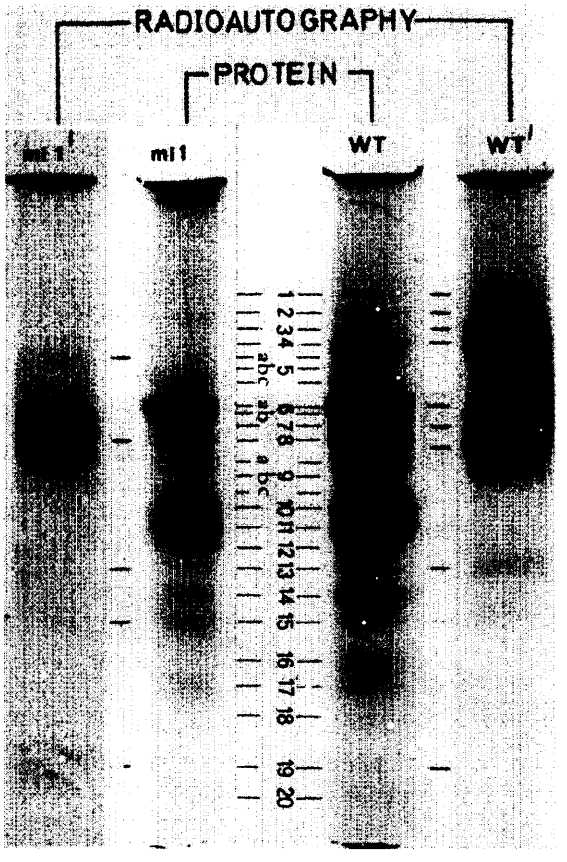

Fig. 2. Electrophoretic patterns of IMP from wild type (WT) and mi-1. The gel was loaded with 100 μg wild type IMP and about 70 μg mi-1 IMP. Inward amido black staining. Outward radioautographs. WT pattern containing 10 000 cpm on the gel was exposed for 1 week, mi-1 containing 600 cpm for 4 weeks.

A similar pattern of labelling *in vitro* is also found in rat liver [8] and in locust flight muscle mitochondria (Sebald, unpublished data). In the IMP of the mi-1 mutant only band 8 is labelled to a higher extent (20% of the total radioactivity). Bands 5a, 13, 15 and 19 exhibit low radioactivity. The 'difference profile' of radioactivity (wild type IMP minus mi-1 IMP, total radioactivity = 100%) has been superimposed on the difference profile of protein in fig. 4. Small changes in protein content are accompanied by big changes in radioactivity in bands 4, 6a, 13 and 19. If all protein fractions labelled *in vitro* are of the same specific radioactivity, they would amount to about one tenth of total IMP or to about 5% of the total mitochondrial protein. This assumption seems to be reasonable since

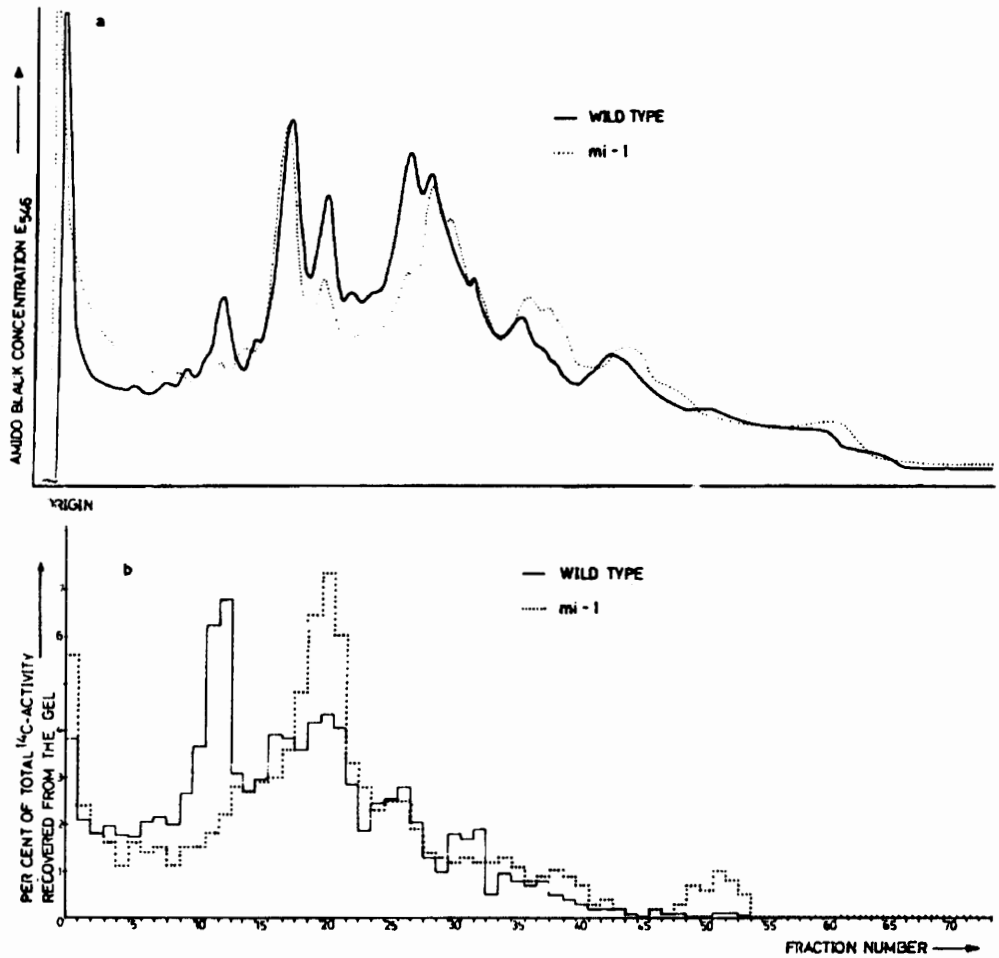

Fig. 3. (a) Densitogramms of wild type and mi-1 IMP after electrophoresis (total expressed as 100%). (b) Distribution of radioactivity in the pherogramms shown in (a) (total as 100%).

mitochondria growing *in vivo* show (1) constant proportion of the enzyme pattern [15] and (2) homogenous labelling of all insoluble protein components after electrophoretic separation [8].

Our results may be summarized as follows:

1. Mitochondria *in vitro* synthesize defined proteins which are identical to those produced *in vivo*: After electrophoretic separation of IMP isolated from mitochondria labelled *in vitro*, bands can be distinguished by autoradiographic techniques coincident with the protein bands formed by IMP synthesized *in vivo* (fig. 2).

2. Mitochondria from mi-1 supplied with ATP under same experimental conditions as mitochondria from wildtype *in vitro* incorporate amino acids. This suggests that by the mutation from wildtype to mi-1 the protein synthesizing system of mitochondria was left qualitatively unaffected. Quantitative differences in the incorporation rate remain to be clarified.

3. In mi-1, single products of the protein synthesizing system of mitochondria differ from those produced in wildtype mitochondria. Some of them are missing or produced in lower quantities.

4. With the reservations mentioned above the products of mitochondrial protein synthesis amount to only 5% of the total mitochondrial protein. Hence a single 'structural protein' [14] described to form 20% [5] or more [14] of whole mitochondrial protein cannot be synthesized by the mitochondrion itself. Furthermore the bands mainly labelled in IMP of

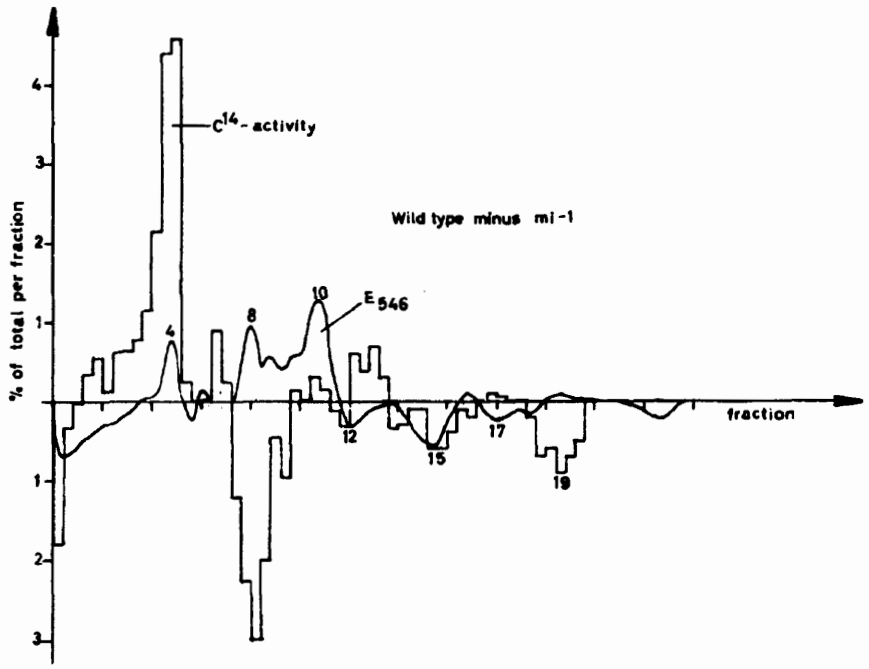

Fig. 4. 'Difference profiles' of wild type minus mi-1 IMP evaluated from the pherogramms in figs. 3a and 3b. Smooth line: protein, broken line: radioactivity. Differences were calculated as per cent of total for the fractions indicated by steps of the radioactivity curve.

wild type and mi-1 differ from those which are enriched in preparations of 'structural protein' from wild type (bands 10 and 11 in fig. 2). The experimental basis of Woodward and Muncres' explanation for the 'pleiotropic phenotype' of mi-1 is not supported by our data.

5. In *Neurospora crassa* by direct evidence it is demonstrated that cytoplasmically inherited alterations from wild type *in vitro* can be correlated to functional deviations of biosynthetic activities in mitochondria isolated from living cells.

Acknowledgements

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