AN ALTERED CYTOCHROME OXIDASE IN A CYTOPLASMIC MUTANT OF *NEUROSPORA*

David L. EDWARDS and Dow O. WOODWARD
Department of Biological Sciences, Stanford University, Stanford, California 94305, USA

Received 11 July 1969

1. Introduction

Mitchell and Mitchell [1] have described a prototrophic, respratory-deficient mutant of *Neurospora* called mi-1 or poky. This mutant differs from wild-type in colony morphology, growth pattern on sucrose, and in the cytochrome content of its mitochondria. In comparison with wild-type levels, poky mitochondria have an excess of cytochrome *c* and are deficient in cytochromes *b* and *a* [2,3]. Sexual crosses with poky have shown that the mutation does not segregate in a Mendelian manner but is inherited through the maternal cytoplasm and is therefore due to an alteration in the cytoplasmic genetic material [1,2]. This observation has been confirmed in our laboratory by analysis of tetrads isolated from sexual crosses with poky [4]. To account for the observed respiration of this mutant in the absence of cytochromes *b* and *a*, an alternate respiratory chain of undetermined composition has been proposed [5,6]. We have observed that mitochondria isolated from the poky strain have cytochrome oxidase activity although wild-type cytochrome *a* is missing. This report deals with the isolation and properties of the altered cytochrome oxidase.

2. Experimental

Strains used in these studies were obtained from the Fungal Genetics Stock Center (FGSC), Hanover, N.H. The wild-type strain used was STA4 (FGSC No. 262). The poky strain was mi-1-1.4 (FGSC No. 1575).

Cultures of *Neurospora* were grown with aeration in 15 l carboys on Vogel's minimal media with 2% sucrose. Wild-type cultures were harvested after 48 hr. Poky cultures were harvested after 96 hr. Mitochondria were prepared from mycelia by a modification of the method of Greenawalt et al. [7].

Cytochrome oxidase was prepared from isolated mitochondria in the following manner: Wild-type or poky mitochondria were suspended in 0.25 M sucrose, 25 mM Tris-Cl, 0.5 mM EDTA, pH 7.5 to a concentration of about 20 mg/ml, mixed with $\frac{1}{3}$ volume of 0.9% KCl and frozen overnight. The mitochondria were then thawed, centrifuged at 34,000 × *g* for 15 min and the supernatant discarded. The pellet was suspended to a concentration of 10–15 mg/ml in 0.66 M sucrose, 50 mM Tris-Cl, mM EDTA, pH 8. The suspension was then fractionated with deoxycholate in the presence of 1 M KCl. The material solubilized between 0.2 and 1.0 mg deoxycholate/mg protein was retained and dialyzed for 2 hr against 10 mM Tris-Cl, pH 8. The dialyzed preparation was then fractionated with neutral ammonium sulfate. The poky enzyme was recovered in a 12–16% ammonium sulfate fraction while the wild-type enzyme appeared in a 16–20% fraction. Preparations were refractioned with ammonium sulfate and then dissolved in a small volume of 10 mM Tris-Cl, 0.1% deoxycholate, pH 8 and stored frozen.

Cytochrome oxidase activity was measured by the spectrophotometric method of Smith [8]. A lineweaver-Burk plot was used to determine initial velocities at infinite cytochrome *c* concentrations. Ferrocytochrome *c* was prepared by the method of Yonetani [9]. Heme *a* was determined by the method of Morrison

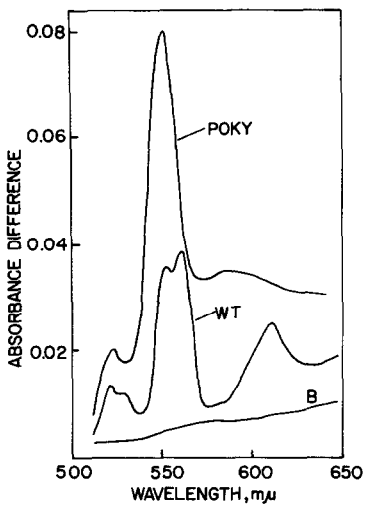

Fig. 1. Difference spectra between oxidized and dithionite-reduced mitochondria from wild-type and poky. Mitochondria were suspended in 0.1 M Tris-Cl pH 8 and solubilized with 2 mg deoxycholate/mg protein. The solution was clarified by centrifugation at 34,000 X *g* for 15 min. Reduction was with dithionite. B is the oxidized versus oxidized baseline.

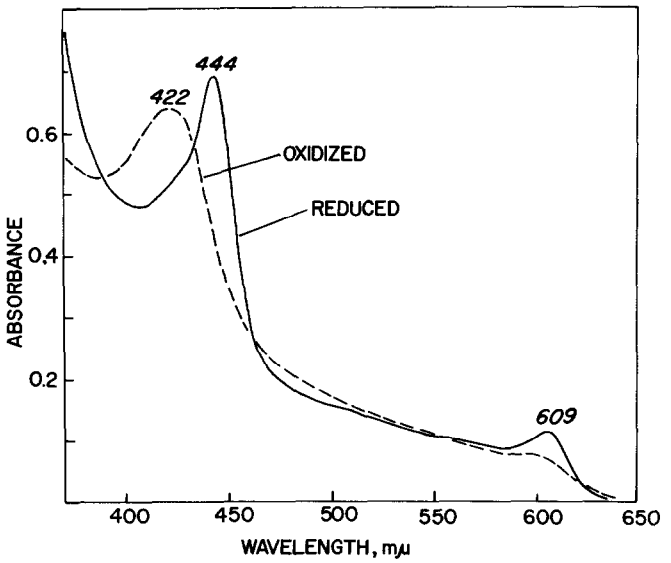

Fig. 2. Absorption spectra of the purified cytochrome oxidase from wild-type in 0.01 M Tris-Cl 0.1% deoxycholate pH 8. Reduction is with dithionite.

and Horie [10]. Protein was measured as described by Lowry [11]. Spectra were determined on a Cary Model 15 recording spectrophotometer.

3. Results

Difference spectra of wild-type and poky mitochondria are shown in fig. 1. These are consistent with previous reports on spectral properties of poky [2,3]. Wild-type mithcondria have absorption bands at 550, 560 and 610 mμ, corresponding, respectively, to cytochrome *c*, *b* and *a*. Poky mitochondria have a large *c* absorption band at 550 mμ but are deficient in bands corresponding to *b* and *a*. The absorption spectrum of the purified wild-type enzyme is shown in fig. 2. It is characterized by a Soret band at 422 mμ which shifts to 444 mμ upon reduction with dithionite. An α absorption band at 609 mμ also appears in the reduced form. These spectral properties are typical of cytochrome oxidase preparations from other sources. The absorption spectrum of the purified poky enzyme is shown in fig. 3. The Soret band ap-

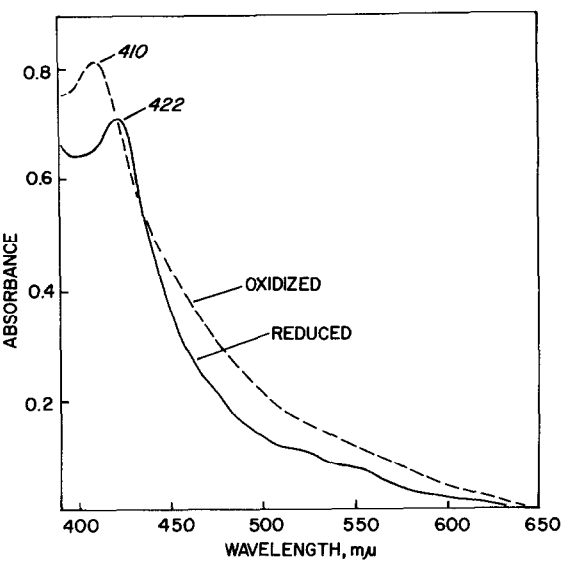

Fig. 3. Absorption spectra of the purified cytochrome oxidase from poky in 0.01 M Tris-Cl 0.1% deoxycholate pH 8. Reduction is with dithionite.

Table 1
Cytochrome oxidase activities.

Preparation	Activity* μmoles/min/mg
Wild-type mitochondria	0.9
Wild-type cytochrome oxidase	25.8
100°/1 min	0
Purification	28.6
Poky mitochondria	0.11
Poky cytochrome oxidase	1.6
Poky cytochrome oxidase + 50 units catalase	1.7
100°/1 min	0
Purification	14.5

* Activities were determined in 0.1 M phosphate buffer pH 6.5. Initial velocities were determined at various cytochrome *c* concentrations and a Lineweaver-Burk plot was used to determine the initial velocity at infinite cytochrome *c* concentration.

appears at 410 mμ in the oxidized form and shifts to 422 mμ upon reduction with dithionite. No α absorption band is seem in the reduced form. This altered spectrum found in poky is a maternally inherited characteristic based on our tetrad analysis [12].

Both preparations have cytochrome *c* oxidase activity. The reaction in each case is first-order with respect to ferrocytochrome *c* concentration. The rate of the reaction is not altered by the presence of either catalase or hydrogen peroxide in the reaction medium. Representative enzyme activities are shown in table 1. The presence of heme *a* in both the wild-type and poky enzymes was demonstrated by extracting the heme from each preparation with acid-acetone and determining the spectrum of the reduced pyridine hemochromagen derivative. The resultant spectra had α absorption maxima at 583 mμ. This value is characteristic of an *a*-type heme [13]. Heme *b* was also present in the pyridine hemochromogen preparation from poky as evidenced by an α absorption band at 557 mμ that was not present in the wild-type preparation. This is characteristic of a *b* type heme [13].

4. Discussion

The absorption spectrum we report for the poky enzyme is not unique to our studies and has been reported earlier by Polis and Shmukler [14] for a compound they called mitochrome which they has isolated from aged rat liver mitochondria. This compound had absorption maxima at 410 and 422 mμ in the oxidized and reduced forms respectively. It was later shown to arise, with loss of enzymatic activity, from normal cytochrome oxidase preparations that were aged in the cold [15]. A mechanism for this alteration in spectral properties has been described by Lemberg [16] which involves a conformational change in the protein moiety of the enzyme due to denaturation and the resultant formation of an aldimine linkage between the formyl group of heme *a* and the ε amino group of a lysine residue of the protein. We believe that the altered spectral properties in our preparation from poky do not arise due to a denaturation process as enzymatic activity is retained (although with a lower specific activity than wild-type) and the altered Soret band can be observed in preparations of whole mitochondrial membranes from poky. The presence of heme *a* in both enzyme preparations, however, suggests that the alteration in poky does lie in the protein moiety of the enzyme rather than in the heme group. A protein component of altered configuration due to a genetic change could result in the altered spectral properties observed by an aldimine-type mechanism similar to the one described by Lemberg for mitochrome.

The presence of heme *b* in the preparation from poky may account for the lower purification factor of the poky enzyme as compared with wild-type (table 1). Elliot et al. [15] have shown that preparations of cytochrome *b* also revert to the mitochrome spectrum on aging. This preparation that we isolate from poky which has spectral properties that are not characteristic of either cytochrome *a* or cytochrome *b* but contains both heme *a* and heme *b* may therefore account for the observed deficiency of both of these cytochromes in poky.

The presence of DNA in *Neurospora* mitochondria is well documented [17]. It is likely that the gene responsible for the alteration in cytochrome oxidase

is in this mitochondrial DNA. Clark-Walker and Linnane have studied the effects of chloramphenicol on yeast cytochromes and have concluded that cytochrome *a* is synthesized within the mitochondrion [18]. Reports conflicting to this suggestion have also appeared, however, and suggest that cytochrome *a* is synthesized on cytoplasmic ribosomes [19,20]. Recent studies have shown that cytochrome oxidase is composed of two major protein components [21, 22]. To account for the observed genetic results in *Neurospora*, we must conclude that at least one of these proteins is either specified by a cytoplasmic gene or directly influenced by a cytoplasmic gene product. Studies as to the nature of the components of cytochrome oxidase and their genetic origins are continuing in our laboratory.

Acknowledgements

This work was supported by grants from the National Science Foundation and the National Institutes of Health of the United States. It was carried out while D.L.E. was the recipient of a U.S. Public Health Service Postdoctoral Fellowship.

References

[1] M.Mitchell and H.Mitchell, Proc. Natl. Acad. Sci U.S. 38 (1952).
[2] M.Mitchell, H.Mitchell and A.Tissieres, Proc. Natl. Acad. Sci. U.S. 39 (1953) 606.
[3] A.Griffiths, H.Bertrand and T.Pittenger, Neurospora Newsletter 13 (1968) 16.
[4] M.Grindle, unpublished data.
[5] A.Tissieres, H.Mitchell and F.Haskins, J. Biol. Chem. 205 (1953) 423.
[6] R.Eakin, Ph. D. Thesis, Calif. Institute of Technology (1968).
[7] J.Greenawalt, D.Hall and O.Wallis, in: Methods in Enzymology, vol. 10, eds. R.Estabrook and M.Pullman (Academic Press, New York, 1967) p. 142.
[8] L.Smith, Methods of Biochemical Analysis, vol. 2 (Interscience, New York, 1955) p. 427.
[9] T.Yonetani, in: Biochemical Preparations, vol. 11, ed. A.Maehly (John Wiley, New York, 1966) p. 14.
[10] M.Morrison and S.Horie, Anal. Biochem. 12 (1965) 77.
[11] O.Lowry, N.Rosenberg, A.Farr and R.Randall, J. Biol. Chem. 193 (1951) 265.
[12] D.Edwards, M.Grindle and D.Woodward, Fed. Proc. 28 (1969) 414.
[13] P.Nicholls, in: The Enzymes, vol. 8, eds. P.Boyer, H. Lardy and K.Myrback (Academic Press, New York, 1963) p. 35.
[14] B.Polis and H.Shmukler, J. Biol. Chem. 227 (1957) 419.
[15] W.Elliot, W.Hulsmann and E.Slater, Biochem. Biophys. Acta 33 (1959) 509.
[16] M.Lemberg, Physiol. Revs. 49 (1969) 48.
[17] E.Reich and D.Luck, Proc. Natl. Acad. Sci. U.S. 55 (1966) 1600.
[18] G.Clark-Walker and A.Linnane, J. Cell Biol. 34 (1967) 1.
[19] B.Kadenbach, Biochem. Biophys. Acta 132 (1966) 430.
[20] H.Schieffer, Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 235.
[21] H.Tuppy and G.Birkenmeyer, European J. Biochem. 8 (1969) 237.
[22] F.Sun, K.Prezbindowski, F.Crane and E.Jacobs, Biochem. Biophys. Acta 153 (1968) 804.