Biochimica et Biophysica Acta, 936 (1988) 133–138
Elsevier

BBA 42855

Myxothiazol resistance in human mitochondria

William Davis Parker, Jr. [a], Frank Frerman [a], Richard Haas [b] and Janice K. Parks [a]

[a] *The University of Colorado School of Medicine, Denver, CO and* [b] *The School of Medicine, University of California at San Diego, San Diego CA (U.S.A.)*

(Received 28 April 1988)

Key words: Cytochrome *b*; Quinol oxidase; Myxothiazol; Electron transfer; Lactic acidosis; (Human mitochondria)

We have investigated electron transfer activities of respiratory chain complexes in platelet mitochondria of a patient with intermittent ataxia and lactic acidosis who was previously reported to be deficient in the E_1 (decarboxylase) component of the pyruvate dehydrogenase complex. Electron transfer from succinate to cytochrome *c* was normal, but the mitochondria exhibited moderately decreased (63% of control) quinol : cytochrome-*c* oxidoreductase activity, suggesting a defect in complex III. Consistent with some perturbation in complex III, electron flux through complex III was resistant to inhibition by myxothiazol compared to normal controls. In contrast, titration with antimycin revealed a less abnormal pattern of inhibition. The extreme specificity of myxothiazol binding at or near the quinol oxidase domain of mitochondrial cytochrome *b*, i.e., *b*-566, suggests a defect in this region of complex III which may perturb the kinetics or thermodynamics of quinol oxidation in the complex. These data suggest that the patient's illness results from a mutation in the quinol oxidase domain of mitochondrial cytochrome *b* (*b*-566).

Introduction

Mechanistic, structural and genetic investigations of the human respiratory chain are still in the developmental stages. A driving force for these investigations is the increasing number of human neurologic diseases that are being recognized as being mitochondrially based. There remain a number of technical problems which must be addressed, not the least of which is the small amounts of material that can be obtained through the two conventional sources of human mitochondria, cultured skin fibroblasts and muscle biopsy. There also exists a major problem in obtaining control tissues from unaffected subjects, this dilemma being especially severe when control muscle mitochondria are needed. Many of the respiratory chain defects that have been reported in humans also involve the absence of more than one protein in a single respiratory complex [1,2], and at times, the accompanying decreases in the mass and/or activities of other complexes. The latter observation, which is quite common, is ample evidence for the need for methodologies to investigate the molecular properties of these oxidation-reduction proteins beyond the simple spectral, catalytic and immunologic levels.

In this paper, we present a method for obtaining large amounts of human mitochondria from platelets obtained by plateletpheresis. This approach has the advantages of providing large amounts of reasonably pure mitochondria from a single preparation. Furthermore this procedure is

Correspondence: W.D. Parker, Box C-233, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262, U.S.A.

relatively noninvasive and can be repeated on a single subject at several day intervals. It also has advantages over human diploid fibroblasts as a source of mitochondria in terms of time needed to obtain adequate yields of mitochondria and in terms of avoiding the problem of fibroblast senescence. We have employed this new method to detect an abnormality in complex III in a patient with a neurologic disorder, intermittent ataxia [3]. This patient was previously reported deficient in the E_1 component of the pyruvate dehydrogenase complex [4]. This is the first report of resistance in humans to inhibition by myxothiazol. This highly specific inhibitor of cytochrome *b* of complex III acts at the *b*-566 heme group of the cytochrome and inhibits ubiquinol oxidation at a concentration stoichiometric with the *b*-566 center [5]. Previous investigations have shown that mutations conferring resistance to inhibition of cytochrome *b* by myxothiazol in higher and lower eucaryotes map exclusively to the mitochondrial cytochrome *b* gene [6,7].

Methods

After approval of the project by the Institutional Review Board and after informed consent by the patient, blood platelets were collected by plateletpheresis. A clinical description of this patient has been published previously [3]. Control platelets were obtained from single volunteer normal controls with no personal or family history of mitochondrial or neurologic disease.

A crude mitochondrial pellet was prepared by a modification of the method of Salganicoff [8,9]. Unless otherwise stated, reagents were obtained from Sigma. EGTA was substituted for EDTA and platelet disruption was accomplished by nitrogen cavitation [10]. The crude mitochondrial pellet was resuspended in 125 mM NaCl/2 mM $CaCl_2$ and held for 5 min at room temperature in order to promote depletion of alpha-granules [11]. The mitochondria were repelleted by centrifugation at $17000 \times g$ for 10 min. These mitochondria were further purified by centrifugation on a Percoll (Pharmacia) gradient [12]. Mitochondria were found in two zones corresponding to densities of 1.054 and 1.096. Densities were determined by centrifugation of density marker beads on a parallel gradient. The mitochondria were recovered from the gradient by careful aspiration, diluted 40-fold in 250 mM sucrose/10 mM Tris/and 1 mM EDTA (pH 7.4) and sedimented by centrifugation at $17000 \times g$ for 10 min. The purified mitochondria were resuspended in a small volume of sucrose/Tris/EDTA, quick-frozen in an acetone/dry ice bath and stored at $-70\,^{\circ}C$.

Purified platelet mitochondria were assayed for NADH : ferricyanide oxidoreductase [13], NADH : ubiquinone oxidoreductase (complex I) using Q_1 [13], succinate : cytochrome-*c* oxidoreductase [14], alpha-glycerol phosphate : cytochrome-*c* oxidoreductase [14], cytochrome-*c* oxidase, ubiquinol : cytochrome-*c* oxidoreductase using Q_1H_2 [15], and succinate dehydrogenase [16].

The concentrations of cytochrome *b* and of cytochrome $a + a_3$ were determined at room temperature from dithionite reduced minus oxidized difference spectra using a Shimadzu UV-3000 spectrophotometer. Reduction was accomplished with dithionite after solubilization using *n*-dodecyl β-D-maltoside (Calbiochem) [17]. Cytochrome concentrations were calculated using the absorption coefficients determined by Rieske [18].

Results

Activities of specific segments of the electron transport chain (Table I) were within control ranges, except for ubiquinol : cytochrome-*c* oxidoreductase activity, which was moderately decreased to 63% of control values.

The 37% reduction of quinol : cytochrome-*c* oxidoreductase activity led us to focus on the three redox-active proteins in complex III which can be measured by visible or EPR spectroscopy. Dithionite reduced minus oxidized difference spectra from the patient and a normal control are shown in Fig. 1. The calculated cytochrome *b* : cytochrome $a + a_3$ ratio in the patient was 1.1 and was 1.40 in controls. The slight reduction in the patient is probably not significant. Another property of complex III that reflects the structure-function relationship is the inhibition of electron transport through the complex by myxothiazol and antimycin. Complex III activity can be titrated by either of these tightly binding inhibitors, such

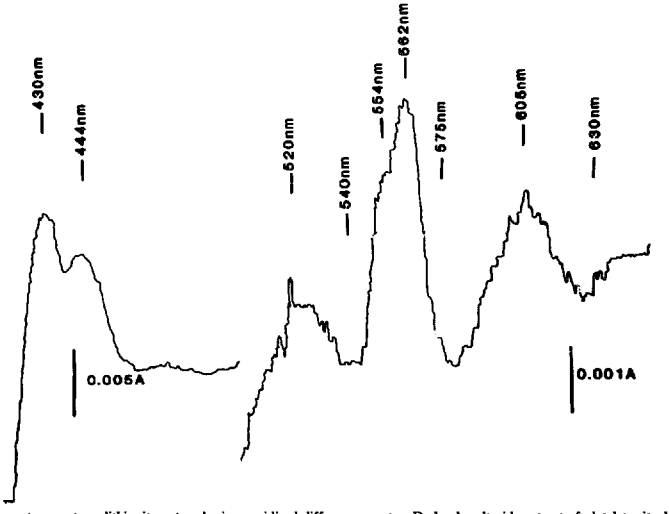

Fig. 1. Room temperature dithionite reduced minus oxidized difference spectra. Dodecyl maltoside extract of platelet mitochondria was assayed at a mitochondrial protein concentration of 3 mg/ml.

that complete inhibition is achieved at an inhibitor : cytochrome-*b* ratio of 1 : 2, based on total cytochrome *b* chromophores [19]. Myxothiazol is thought to bind at or near the cytochrome *b*-566 center, which, in the Q-cycle model of complex III, has quinol oxidase activity [20]. Antimycin perturbs the absorption spectrum of the high-potential cytochrome *b*-562 center, the quinone reductase center, and by inference, binds at or near this chromophore. Any difference in the binding of these inhibitors might reveal a strucutral abnormality in the complex. This may be particularly true of myxothiazol, which has been proposed to act as a ubiquinone analog [19,21]. Electron transport through complex III was titrated with myxothiazol and antimycin using α-glycerol phosphate as the reductant, since previous studies had indicated a relatively high activity of this reductase of complex III in platelet mitochondria [9]. Further, the topography of the protein obviates the necessity of disrupting the mitochondria, which could result in minor dif-

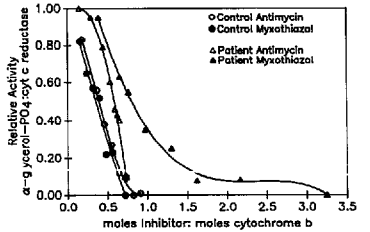

Fig. 2. Inhibitor titrations of alpha-glycerol phosphate : cytochrome-*c* oxidoreductase activity.

TABLE I

ASSAYS OF RESPIRATORY CHAIN ACTIVITIES IN ISOLATED PLATELET MITOCHONDRIA

Activities are measured as nmol/min per mg.

Complex I	NADH-ubiquinone oxidoreductase	
	total	rotenone-sensitive
control ($n = 7$)	24.4 ± 7.4	18.2 ± 6.4
patient	31.5	19.4
Complexes II and III	succinate: cytochrome-*c* oxidoreductase	ubiquinol: cytochrome-*c* oxidoreductase
control	217.0 ± 115.0 ($n = 6$)	351.9 ± 146.23 ($n = 4$)
patient	137	125.2
Complex IV	cytochrome-*c* oxidase	
control ($n = 6$)	135.3 ± 39.2	
patient	171	

ferences in membrane sidedness among preparations if other dehydrogenases were used as the source of electrons. Results of these experiments are shown in Fig. 2. Control data from four normal controls showed the predicted titration of activity, with both inhibitors at a stoichiometry of 1:2 (inhibitor: cytochrome *b*), and the titration curves are monotonic. In marked contrast, electron transfer through complex III in the patient was relatively resistant to inhibition by myxothiazol and to a lesser extent to inhibition by antimycin (Fig. 2). The myxothiazol titration curve was shifted to the right and flattened. These effects indicate a difference in binding of inhibitor at the quinol oxidase site. Complete inhibition was not observed even at a 3:1 molar ratio of myxothiazol to cytochrome *b*.

Discussion

The experiments presented here clearly show the utility of (a) platelet mitochondria in the study of human mitochondrial function and (b) the utility of titrations with highly specific, tightly binding (i.e., $K_d \approx 10^{-12}$ M) inhibitors. Our preparations are comparable to other mammalian platelet mitochondria [8,9] and, for the study of human mitochondria, have the advantage of being obtained through a relatively noninvasive, inexpensive, rapid procedure in large amounts. The major contaminants of mitochondria prepared in this fashion are unlysed alpha-granules but these do not interfere with spectral or enzymatic assays. The limitation of this source of human mitochondria is the same as that encountered with any other single source of mitochondria, namely, tissue-specific expression of some nuclear genes encoding mitochondrial proteins [22,23]. However, for mutations affecting non-tissue-specific proteins or those encoded on the mitochondrial genome, platelets provide a readily avialable source of human mitochondia. Further, since platelets are nonnucleated, they should provide a rich source of human mitochondrial DNA for studies of the molecular genetics of mitochondrially encoded proteins.

Our data show that there is considerable resistance to inhibition by myxothiazol of electron flux through complex III in the patient's mitochondria. The site of inhibition by myxothiazol is highly specific in eukaryotes. Resistance to myxothiazol inhibition has previously been reported in yeast mutants selected for growth on myxothiazol-containing medium [7]. In these lower eukaryotes, respiration was resistant to myxothiazol inhibition in three different isolates and the mutation was mapped to the *N*-terminal region of the mitochondrial cytochrome *b* gene in all three mutants. In these mutants, the cytochrome *b* content was not significantly different from wild-type strains, as is the case with the patient, and the rate of NADH oxidation in the absence of inhibitor was reduced only 20–40% as compared to the wild type. In the three yeast mutants, the myxothiazol concentration required for 50% inhibition varied from 4–50-fold greater than the wild type. In the patient investigated here, the myxothiazol concentration needed for 50% inhibition is difficult to quantitate, since the curve appears to be biphasic, but is clearly displaced form the control curve, which is monotonic. This biphasic nature of the curve seen with patient's mitochondria is consistent with heteroplasmy for the mitochondrial cytochrome *b* gene, resulting in the superimposition of a curve from normal cytochrome *b* and an abnormal curve produced by mutated cytochrome

b. Howell et al. [6] have described mouse fibroblast cell lines which are resistant to inhibition by myxothiazol. The specific activity of succinate : cytochrome-*c* oxidoreductase is about 60% of that in the control lines, and the concentration of myxothiazol required for 50% inhibition is at least three orders of magnitude greater than the control lines. Thus, even in eukaryotes selected for resistance to inhibition by myxothiazol, the activity of complex III is not drastically reduced as a result of the mutation, paralleling the case with the patient studied here.

The altered component of complex III conferring myxothiazol resistance in the patient has not been unequivocaly demonstrated; however, in yeast and in the mouse fibroblast system, the mutations conferring myxothiazol resistance have always mapped to specific sites encoding the N-terminal region within the mitochondrial cytochrome *b* gene. Nuclear-encoded resistance to myxothiazol resistance has been reported in whole mouse cell fibroblast lines, but these cell lines were also resistant to inhibition by numerous other respiratory chain inhibitors, including azide [24]. This observation may have resulted from a defect in complex I, such that electron flux was extremely low through the entire respiratory chain, with much of the oxidative metabolism occurring via anaerobic pathways. Resistance to numerous inhibitors can be rationalized by the fact that very few electrons reached the specific sites of inhibition; thus, the presence of inhibitors did not produce effects on growth of the cells in culture. Mitochondria from the patient investigated behaved normally toward inhibitors other than myxothiazol (cyanide, rotenone, azide, antimycin). Thus, we conclude that the primary defect in this patient is likely to be in the mitochondrial gene encoding cytochrome *b*. Cytochrome-*c* oxidase activity is increased in the patient's mitochondria, a frequent observation, which has been interpreted as a compensatory physiologic response [1,25].

The physiologic significance of myxothiazol resistance in the patient is not immediately evident. The specific activity of ubiquinol oxidase is reduced about 70%. If this does indeed reflect a mutation at the quinol oxidase site of complex III, and since the total amount of cytochrome *b* is relatively unaffected, the mutation could affect the kinetic or thermodynamic properties of the *b*-566 center. The basis for assignment of this patient's defect to cytochrome *b* is based on the resistance of the patient's mitochondria to inhibition by myxothiazol, a well-characterized inhibitor of cytochrome *b*-566. The observed catalytic abnormalities involving cytochrome *b* and spectrophotometric determinations of cytochrome *b* concentration are secondary to the major argument. The fact that our patient's cytochrome *b* exhibited abnormal responses to myxothiazol, which binds to cytochrome *b* itself (inferred from the red-shift in the 566 nm absorbance of the reduced cytochrome [26]), represents a functional abnormality which must be primary. Our data taken in isolation do not absolutely rule out a mutation in the Rieske iron-sulfur protein: this protein may share a common myxothiazol binding site with cytochrome *b* [5]; however, since myxothiazol resistance studied at the level of the electron transport chain has not been related to iron-sulfur protein mutations and has been invariably related to cytochrome *b* mutations, myxothiazol resistance is strong evidence for a primary defect in cytochrome *b*. The fact that the patient's cytochrome *b* was also mildly resistant to inhibition by antimycin is further argument in favor of the defect residing in cytochrome *b* rather than the iron-sulfur protein. The patient's mild catalytic defect at the *b*-566 site as measured by QH_2 oxidase activity is in keeping with the mild nature of his disease; he becomes symptomatic only at times of metabolic stress, when the electron flux through cytochrome *b* is presumably at its highest.

While spectrohotometrically determined cytochrome *b* was slightly decreased with respect to cytochrome $a + a_3$ as compared to controls, this observation does not prove that the cytochrome *b* molecules itself is defective. Apparent deficiency of substrate-reducible cytochrome *b* has been previously observed in a patient with a mitochondrial myopathy [27], but catalytic abnormalities were not identified. This deficiency of substrate-reducible cytochrome *b* was not necessarily primary, and could have been the result of abnormalities proximal to cytochrome *b* in the electron transport chain, since generation of the reduced state was accomplished through substrate rather than dithionite reduction.

Acknowledgements

Dr. Daniel Ambruso and the staff of the Belle Bonfils Blood Center provided enormous assistance in the collection of platelets. This work was supported by a grant (RR-69) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health, by NIH grants NS24872 (WDP) and DK37749 (FEF), and by a Mental Retardation Research Center Grant from NICHD. W.D.P. and R.H. are recipients of Clinician Investigator Development Awards from NINCDS.

References

1 Darley-Usmar, V.M., Kennaway, N.G., Buist, N.R.M. and Capaldi, R.A. (1983) Proc. Natl. Acad. Sci. USA 80, 5103–5106.
2 Kennaway, N.G., Buist, N.R.M., Darley-Usmar, A., Papadimitrou, S., DiMauro, S., Kelly, R.I., Capaldi, R.I., Blank, N.K. and Agostino, A. (1984) Pediatr. Res. 18, 991–999.
3 Blass, J.P., Kark, R.A.P. and Engel, W.K. (1971) Arch. Neurol. 25, 449–460.
4 Blass, J.P., Avigan, J. and Uhlendorf, B.W. (1970) J. Clin. Invest. 49, 423–432.
5 Von Jagow, G., Ljungdahl, P.O., Graf, P., Ohnishi, T. and Trumpower, B.L. (1984) J. Biol. Chem. 259, 6318–6326.
6 Howell, N., Appel, J., Cook, J.P., Howell, B. and Houswirth, W.W. (1987) J. Biol. Chem. 262, 2411–2414.
7 Thierbach, G. and Michaelis, G. (1982) Mol. Gen. Genet. 186, 501–506.
8 Salganicoff, L. and Fukami, M.H. (1972) Arch. Biochem. Biophys. 153, 726–735.
9 Salganicoff, L., Hebda, P.A., Yandrasitz, J. and Fukami, M.H. (1975) Biochim. Biophys. Acta 385, 394–411.
10 Broekman, M.J., Westmoreland, N.P. and Cohen, P. (1974) J. Cell. Biol. 60, 507–519.
11 VanderMeulen, J. and Grinstein, S. (1982) J. Biol. Chem. 257, 5190–5195.
12 Mickelson, J.R., Greaser, M.L. and Marsh, B.B. (1980) Anal. Biochem. 109, 255–260.
13 Hatefi, Y. (1978) Methods. Enzymol. 53, 11–15.
14 Stumpf, D.A. and Parks, J.K. (1981) Biochem. Med. 25, 234–238.
15 Trumpower, B.L. and Edwards, C.A. (1979) J. Biol. Chem. 254, 8697–8706.
16 Krebs, J.J.R., Hauser, H.and Carafoli, E. (1979) J. Biol. Chem. 254, 5308–5316.
17 Yang, X. and Trumpower, B.L. (1986) J. Biol. Chem. 261, 12282–12289.
18 Rieske, J.S. (1968) Methods. Enzymol. 10, 488–493.
19 Thierbach, G. and Reichenbach, H. (1981) Biochim. Biophys. Acta 638, 282–289.
20 Trumpower, B.L. (1981) Biochim. Biophys. Acta 639, 129–155.
21 Becker, W.F., Von Jaegow, G., Anke, T. and Steglich, W. (1981) FEBS Lett. 132, 329–333.
22 Schulthesiss, H.P. and Klingenberg, M. (1985) Arch. Biochem. Biophys. 239, 273–279.
23 Merle, P. and Kadenbach, B. (1982) Eur. J. Biochem. 125, 239–244.
24 Howell, N. and Nalty, M.S. (1987) Somat. Cell Genet. 13, 529–537.
25 Land, J.M., Morgan-Hughes, J.A. and Clark, J.B. (1981) J. Neurol. Sci. 50, 1–13.
26 Von Jagow, G. and Engel, W.D. (1981) FEBS Lett. 136, 19–24.
27 Morgan-Hughes, J.A., Darveniza, P., Kahn, S.N., Landon, D.N., Sheratt, R.M., Land, J.M. and Clark, J.B. (1977) Brain 100, 616–640.