

Kinetic alterations of cytochrome-*c* oxidase in cystic fibrosis

Maurizio Battino, Michela Rugolo, Giovanni Romeo* and Giorgio Lenaz⁺

*Lab. di Biochimica, Istituto Botanico, Universita' di Bologna, Via Irnerio 42, 40126 Bologna and *Istituto G. Gaslini, Genova, Italy*

Received 5 February 1986

We compared the kinetics of cytochrome-*c* oxidase (cytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1) in fibroblasts derived from normal and cystic fibrosis individuals. The K_m of the enzyme for reduced cytochrome *c* was significantly increased in CF cells; the change, however, was observed only at temperatures above 25°C. The V_{max} values were comparable in both types of individuals.

Cystic fibrosis Fibroblast Mitochondria Cytochrome-c oxidase Cytochrome c

1. INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive exocrinopathy, characterized by chronic pulmonary obstruction and infection, gastrointestinal malabsorption due to pancreatic insufficiency and increased sweat salinity [1,2]. Its incidence is one in 1600 live births in the white population of European descent, making it the most frequent autosomal recessive disorder. Consanguinity studies indicate that this relatively common disease is not due to mutations residing in more than one gene [3]. This gene has been recently mapped on the long arm of chromosome 7 by means of closely linked DNA polymorphisms [4–6] and no genetic heterogeneity has been observed so far by linkage studies of CF families. The basic molecular defect of the disorder is still unknown, although several metabolic, presumably secondary, abnormalities have been reported in the past 30 years.

Significant contributions in the study of CF have been obtained through electrophysiological studies, showing that epithelia from CF patients exhibit lower permeability to chloride ions than those from normal tissues [7,8]; furthermore, it has been shown in our laboratory that Cl^-

transport in cultured fibroblasts from CF patients is significantly lower than in control fibroblasts [9]. Another approach to the understanding of the molecular defect in CF has been the investigation of mitochondrial function. Feigal and Shapiro [10] have shown that mitochondrial calcium uptake is increased in CF fibroblasts, and related this change to an alteration in the kinetic properties of NADH dehydrogenase [11]; in more readily accessible white blood cell preparations however, Sanguinetti-Briceno and Brock [12] failed to reproduce the findings of Shapiro et al. [11].

We have therefore started a systematic study of mitochondrial functions in CF fibroblasts. In this paper we report a preliminary investigation of cytochrome-*c* oxidase (cytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1), a mitochondrial marker enzyme which can be determined when present in small amounts in total homogenates because of its high activity. We present evidence indicating that in CF fibroblasts the K_m of the enzyme for the substrate cytochrome *c* is significantly increased at temperatures above 25°C.

2. MATERIALS AND METHODS

Skin fibroblasts, derived from biopsies of the upper arm, were cultured in Dulbecco's modified

⁺ To whom correspondence should be addressed

Eagle's medium containing 10% fetal calf serum, L-glutamine and antibiotics (penicillin, 100 units/ml; streptomycin, 100 $\mu\text{g/ml}$) in a humidified atmosphere of 5% CO_2 in 95% air at 37°C. Cells were removed from stock flasks by trypsinization and were allowed to grow to confluency in 90 \times 150 mm roller bottles. After washing the cells three times with Hanks' balanced salt solution at pH 7.3, they were collected using a rubber scraper, centrifuged at 1000 rpm for 10 min and resuspended in 25 mM K phosphate, 1 mM EDTA, pH 7.5. The suspension was gently homogenized in the same medium at 0°C with a Potter homogenizer equipped with a teflon pestle and centrifuged at 105000 $\times g$ for 1 h in a Beckman L5-50 ultracentrifuge. The pellet was resuspended in a small aliquot of the same medium at a protein concentration ranging between 3 and 12 mg/ml, as determined according to Lowry et al. [13]. The suspension was frozen and thawed at least 3 times before assay of cytochrome-c oxidase, since the activity was found to increase to a steady level by 2 or 3 freeze-thaw cycles.

Cytochrome-c oxidase activity was assayed at 30°C, unless otherwise stated, in the same buffer, using reduced cytochrome *c* (Sigma, horse heart type IV; reduced by dithionite and purified on a Sephadex G-25 column) as substrate, by monitoring the absorbance decrease of cytochrome *c* upon oxidation at 417–409 nm in a Sigma ZWS dual wavelength spectrophotometer equipped with a rapid mixing apparatus of our design (mixing time ~ 200 ms). The extinction coefficient used was 40.7 $\text{mM}^{-1}\cdot\text{cm}^{-1}$. The cytochrome-c oxidase activity was 100% inhibited by 1 μM KCN.

3. RESULTS

The cytochrome-c oxidase activity of fibroblast homogenates follows saturation kinetics by increasing the reduced cytochrome *c* levels up to a concentration of 8 μM ; above this level substrate inhibition was observed in accordance with other observations [14]. In the range 0.5–8 μM cytochrome *c*, Lineweaver-Burk plots were linear with correlation coefficients better than 0.98. The K_m for reduced cytochrome *c* of cytochrome-c oxidase from normal fibroblasts varied with different cell cultures, ranging between 1.5 and 4.5 μM , although the values were consistently uniform in

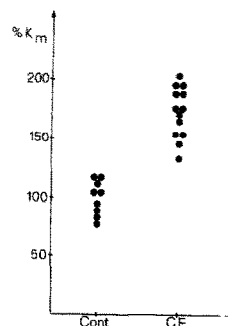


Fig.1. K_m values of cytochrome-c oxidase for cytochrome *c* in 13 CF and 9 control fibroblast lines. The K_m values were determined in 3 separate experiments. In every experiment, the increase in K_m values of CF fibroblasts was statistically significant ($p < 0.02$); K_m values of CF cells are expressed as % of average value obtained in control cells, considered as 100%.

experiments with cell lines from different individuals but cultured in the same experimental set; the V_{\max} values ranged between 12.9 and 20.9 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. We determined the kinetics of cytochrome-c oxidase from 13 CF lines and 9 control lines. Fig.1 compares the K_m of the enzyme in normal and CF cells cultured in the same experimental set. The average value of the K_m was increased by 73% in CF cells with respect to controls; no significant change, however, was found for the V_{\max} .

Fig.2 shows an Arrhenius plot of cytochrome-c oxidase in both normal and CF cells homogenates;

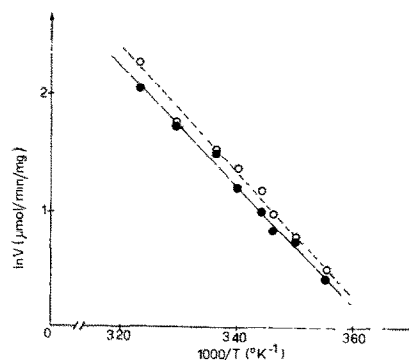


Fig.2. Arrhenius plot of cytochrome-c oxidase in normal (●) and CF (○) fibroblasts. The activation energies were 21 and 23 $\text{kcal}\cdot\text{mol}^{-1}$ for normal and CF fibroblasts, respectively.

the temperature dependence is linear over the range 8.5–36°C, in accordance with other investigations using reduced cytochrome *c* as the substrate [15]. No significant change in the V_{\max} values at any temperature nor in the activation energies was detected between the two systems. The K_m values for reduced cytochrome *c*, however, were found to have a different temperature dependence in normal and CF cells (fig.3). Whereas at lower temperatures (<25°C) the K_m values were not distinguishable between the two systems, at 30 and 37°C the K_m was much higher in CF than in controls.

4. DISCUSSION

This paper shows that in CF fibroblasts the kinetics of cytochrome-*c* oxidase is altered, with a significant increase of the K_m for the substrate, cytochrome *c*. The change, however, is found only at temperatures above 25°C. The alteration described in this investigation adds up to a series of studies showing membrane alterations in CF (review [16]).

Since mutations in a single gene should account for the different biochemical abnormalities reported in CF [3], it seems reasonable to assume that some basic event of cellular function is involved in CF [16]. The present study may offer a clue for the understanding of such a basic defect.

Cytochrome-*c* oxidase is a multisubunit protein complex embedded in the inner mitochondrial membrane [18] and its activity, as for most membrane-bound enzymes [19], is strongly dependent on the lipid environment of the enzymic pro-

tein [20]. In particular, it was found in reconstituted systems that the K_m for cytochrome *c* is affected by the lipid composition of the membrane [20] and that tightly bound cardiolipin is absolutely required for activity [21,22]. The change in K_m and its dependence on temperature might be explained with a change in lipid composition of the mitochondrial membrane. To this purpose, it is interesting that Von Ruecker et al. [17] in a preliminary report have observed a strong decrease of cardiolipin and of the other mitochondrial phospholipids in CF fibroblasts.

The hypothesis that the multiplicity of membrane biochemical alterations in CF may find an explanation in a deranged lipid metabolism is attractive and deserves further investigations.

ACKNOWLEDGEMENTS

This study was supported by a grant from Progetto Finalizzato: 'Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie', CNR, Roma. We wish to thank Dr Mariano Rocchi, Istituto G. Gaslini, Genova, for kindly providing fibroblast lines.

REFERENCES

- [1] Davis, P.B. and Di Sant'Agnese, P.A. (1980) *Pediatr. Res.* 14, 83–87.
- [2] Di Sant'Agnese, P.A. and Davis, P.B. (1976) *N. Engl. J. Med.* 295, 481–485.
- [3] Romeo, G., Bianco, M., Devoto, M., Menozzi, P., Mastella, G., Giunta, A.M., Micalizzi, C., Antonelli, M., Battistini, A., Santamaria, F., Castello, D., Marianelli, A., Marchi, A.G., Manca, A. and Miano, A. (1985) *Am. J. Hum. Genet.* 37, 338–349.
- [4] Knowlton, R.G., Cohen-Haguenaer, O., Van Cong, N., Frezal, J., Brown, V.A., Barker, D., Braman, J.C., Schumm, J.W., Tsui, L., Buchwald, M. and Donis-Keller, H. (1985) *Nature* 318, 380–382.
- [5] White, R., Woodward, S., Leppert, M., O'Connell, P., Hoff, M., Herbst, J., Laluel, J., Dean, M. and Vande Woude, G. (1985) *Nature* 318, 382–384.
- [6] Wainright, B.J., Scambler, P.J., Schmidtke, H., Watson, E.A., Law, H., Farrall, M., Cooke, H.J., Eiberg, H. and Williamson, R. (1985) *Nature* 318, 384–385.
- [7] Quinton, P.M. (1983) *Nature* 301, 421–422.

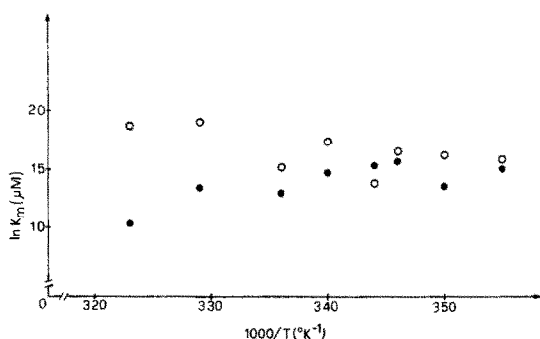


Fig.3. Van 't Hoff plot of cytochrome-*c* oxidase in normal (●) and CF (○) fibroblasts.

- [8] Knowles, M.R., Stutt, M.J., Spock, A., Fischer, N., Gatzky, J.T. and Boucher, R.C. (1983) *Science* 221, 1067–1070.
- [9] Rugolo, M., Romeo, G. and Lenaz, G. (1986) *Biochem. Biophys. Res. Commun.* 134, 233–239.
- [10] Feigal, R.J. and Shapiro, B.L. (1979) *Nature* 278, 276–277.
- [11] Shapiro, B.L., Feigal, R.G. and Lam, L.F.H. (1979) *Proc. Natl. Acad. Sci. USA* 76, 2979–2984.
- [12] Sanguinetti-Briceno, N.R. and Brock, D.J.A. (1982) *Clin. Genet.* 22, 308–311.
- [13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- [14] Yonetani, T. and Ray, G.S. (1965) *J. Biol. Chem.* 240, 3392–3398.
- [15] Sechi, A.M., Landi, L., Bertoli, E., Parenti-Castelli, G., Lenaz, G. and Curatola, G. (1973) *Acta Vitaminol. Enzymol.* 22, 177–190.
- [16] Mangos, J.A. and Boyd, R.L. (1984) in: *Cystic Fibrosis: Horizons* (Lawson, ed.) pp.29–50, Wiley, New York.
- [17] Von Ruecker, A., Bertele, R.M., Harms, H.K., Shin, Y.S. and Endres, W. (1984) in: *Cystic Fibrosis: Horizons* (Lawson, ed.) p.52, Wiley, New York.
- [18] Capaldi, R.A., Malatesta, F. and Darley-USmar, V.M. (1983) *Biochim. Biophys. Acta* 726, 135–148.
- [19] Lenaz, G. (1979) *Subcell. Biochem.* 6, 273–343.
- [20] Zahler, W.L. and Fleischer, S. (1971) *J. Bioenerg.* 2, 209–215.
- [21] Vik, S.B., Georgevich, G. and Capaldi, R.A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 1456–1460.
- [22] Fry, M. and Green, D.E. (1980) *Biochem. Biophys. Res. Commun.* 93, 1238–1246.