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Mitochondrial NADH Dehydrogenase in Cystic Fibrosis: Enzyme Kinetics in Cultured Fibroblasts

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SUMMARY

Differences among cystic fibrosis (CF) genotypes (CF, obligate carriers for CF [HZ], and controls) in mitochondrial calcium pool size, oxygen (O_2) consumption, and rotenone inhibition of O_2 consumption led to examination of mitochondrial NADH dehydrogenase (NADH: [acceptor] oxidoreductase, E.C. 1.6.99.3). pH optima of mitochondrial NADH dehydrogenase were different in enzyme derived from whole cell homogenates of cultured skin fibroblasts of subjects with CF, HZ, and controls. We describe here apparent binding of substrate to the enzyme (K_m [NADH]) in cell fractions. K_m (NADH) for CF ranged from 10.9 to 16.1 μM (no. = 7); for HZ from 20.9 to 26.3 μM (no. = 5). With three exceptions, K_m for controls (no. = 12) ranged from 31.8 to 42.8 μM. K_m of the three exceptional controls were 21.5, 23.7, and 22.4 μM (the latter two are identical twins). pH optima of enzyme from these three strains were no different from that of known HZ. The correlation between two kinetic parameters of an enzyme and the three CF genotypes suggests an association between the CF gene and mitochondrial NADH dehydrogenase.

INTRODUCTION

Cystic fibrosis (CF) is the most prevalent lethal single gene disease in Caucasian populations. It is characterized by chronic pulmonary obstruction and infection, gastrointestinal malabsorption due to pancreatic insufficiency, and increased sweat salinity [1, 2]. CF is transmitted as an autosomal recessive trait and occurs in

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approximately one in 1,600 live births. Based on this estimate, one in 20 healthy individuals is a CF carrier. A major goal of carrier identification prior to the birth of an affected child has not been achieved. The birth of a child affected with CF has been the only sign that both parents are heterozygous carriers (HZ). None of the suggested pathogenetic pathways has been generally accepted. The gene product ultimately responsible for the signs and symptoms of CF has been elusive. What has been sought, as with all Mendelian conditions, is a protein whoe qualitative characteristics are different from normal in homozygotes and heterozygotes for the gene.

CF is thought to be a generalized exocrinopathy. Because fibroblasts are secretory cells and retain the donor's genotype in culture, we chose to use them as a model for CF studies [3]. We observed that in comparison with age-, sex-, and passage-matched controls: (1) cells from CF express premature senescence [4, 5]; (2) cells from CF and HZ possess increased intracellular calcium (Ca) pools [3, 6, 7]; (3) mitochondria are the primary sites of the Ca difference [8]; (4) cells from CF and HZ consume more oxygen [8, 9]; (5) cells from CF and HZ respond differentially in terms of oxygen consumption to the electron transport inhibitor, rotenone [8, 10]; and (6) mitochondrial NADH dehydrogenase (NADH: [acceptor] oxidoreductase, E.C. 1.6.99.3), the site of action of rotenone, has different pH optima in preparations from CF, HZ, and controls [10].

These last studies were carried out with whole cell homogenates using a fluorophotometric assay [10]. Because of nonenzymatic photochemical effects on fluorescence, fluorometry is less than ideal for this reaction. Here we describe kinetics of NADH dehydrogenase from mitochondrial preparations using a spectrophotometric method that is preferable to the fluorometric method used previously. We report here distinctive kinetic properties of mitochondrial NADH dehydrogenase in CF, HZ and controls.

MATERIALS AND METHODS

Skin fibroblasts were obtained (in accord with standards of the Committee on the Use of Human Subjects in Research at this institution) and maintained as described [6]. Three HZ strains were obtained from parents of three of the individuals from whom we obtained CF strains. None of the strains were from sibs. Confluent monolayers from eight 75-cm^2 flasks (Falcon) (approximately 1.5–2 $\times$ 10^6 cells/flask) were harvested with (5 ml/flask) trypsin/EDTA (1:250 trypsin + EDTA at 0.2 g/l [Gibco, Grand Island, N.Y.]) and pooled in two centrifuge tubes with 8 ml/tube of 10% fetal calf serum and MEM medium (Gibco). The cells were centrifuged at 500 *g* for 5 min. The pellet was washed twice and centrifuged at 500 *g* for 5 min at 4°C using 10.0 ml of 0.9% NaCl. At the second wash, an aliquot (100 μl) of cells was removed for counting in a ZBI Coulter Counter. Cell preparations were kept at 4°C for all subsequent steps until enzyme assay. The pellet, suspended in 8 ml of cold sucrose A solution (0.25 M sucrose, 1 mM EDTA [EGTA in later experiments], 10 mM Tris buffer, pH 7.4), was homogenized with 15–20 strokes in a Dounce homogenizer and centrifuged at 600 *g* for 10 min. The supernate was saved for the mitochondrial fraction. The pellet was washed twice in 2 ml of sucrose A solution and centrifuged at 600 *g* for 10 min. This supernate was added to the supernate containing mitochondria and the pellet discarded. The supernate was centrifuged at 20,000 *g* for 15 min. For microsomal preparations, the resultant supernate was spun at 105,000 *g* for 45 min and the pellet retained. For mitochondrial preparations, the 20,000 *g* pellet was washed once in 5 ml of ice cold sucrose

B solution (0.25 M sucrose in 10 mM Tris buffer, pH 7.4) and centrifuged at 20,000 *g* for 15 min. The pellet was suspended in 2 ml of ice-cold 0.03 M potassium phosphate buffer, pH 7.6, and frozen and thawed four times in a dry ice-methanol mixture to disrupt mitochondria and centrifuged at 270 *g* for 5 min. The pellet was discarded. An aliquot of the supernate was used for a protein assay [11] and the remainder used for mitochondrial NADH dehydrogenase assays. The sample was maintained at 4° C. All other solutions were kept at 30° C and the enzyme reaction run at 30° C. Enzyme reactions were run on the day of sample preparation.

Fractions were studied for the presence of mitochondria with a succinate dehydrogenase (SDH) assay [8, 9] and for the presence of microsomes with a glucose-6-phosphatase (G6P'ase) assay [12].

The principle of the NADH dehydrogenase reaction is the oxidation of NADH by sample enzyme using the artificial electron acceptor, potassium ferricyanide [$K_3Fe(CN)_6$] (rotenone does not act as an inhibitor with this acceptor). The reaction is followed spectrophotometrically (Beckman Acta CIII) by observing the change in absorbance due to reduction of $K_3Fe(CN)_6$ at 420 nm. The reaction mixture consists of 300 μl of 1 mM NADH (100 μM in final volume), 300 μl of 1 mM $K_3Fe(CN)_6$ (100 μM in final volume), 2.30 ml of 0.12 M potassium phosphate buffer (pH 7.9), and 100 μl of sample. The sample concentrations were adjusted with 0.03 M phosphate buffer to yield approximately a 0.10–0.15 Δ OD/min [13]. The reaction mixture less the tissue sample was incubated in the spectrophotometer for 1 min. At these concentrations of reagents, no Δ OD was observed prior to addition of enzyme preparation. The sample was injected into the cuvette without removing the cuvette from the spectrophotometer and mixed by depressing and releasing the pipette plunger several times. The Δ OD was recorded during the initial 40–60 seconds, the period in which the reaction is linear [13, 14], and expressed as Δ OD/min per cell or mg protein. Michaelis constants (K_m) were determined by varying NADH concentrations (from 10 to 100 μM). Assays were run in duplicate or triplicate depending on enzyme activity of samples. The number of experiments with different strains varied from 1 to 5 (table 1).

K_m for SDH from mitochondrial preparations were estimated using the spectrophotometric method of King [15] with $K_3Fe(CN)_6$ concentrations from 0.20 to 1.50 mM and 4.0 mM of sodium succinate. K_m for both enzymes systems was estimated from least-squares analyses of double reciprocal plots.

RESULTS

Recovery of protein after fractionations, and relative protein concentrations of the different fractions were similar in preparations from CF and control strains. The specific activity of SDH, a mitochondrial marker, in the 20,000 *g* fractions was more than 35× that in the whole cell homogenates; no SDH activity was detected in the 105,000 *g* pellet [8, 9]. Approximately 15% of whole cell G6P'ase activity, the microsomal marker, was detected in the 20,000 *g* pellet.

With the exception of three control strains, K_m (NADH) of NADH dehydrogenase from individuals in each of the three groups was distinct. The K_m for seven CF strains ranged from 10.9 to 16.1 μM; and for five HZ strains from 20.9 to 26.3 μM. K_m for nine control strains ranged from 31.8 to 42.8 μM. Preparations from #86, a nulliparous control, and #93 and #94, identical twin infant controls, yielded K_m values no different from those observed in known HZ (table 1). pH optima for NADH dehydrogenase activity in these three strains, not reported previously, agreed with those of known HZ [10]. Representative double reciprocal plots of enzyme from the three genotypes are shown in figure 1. Substrate inhibition occurred only with NADH concentrations 2–6× K_m (NADH).

TABLE 1

MICHAELIS CONSTANTS OF NADH DEHYDROGENASE FROM SKIN FIBROBLAST MITOCHONDRIAL PREPARATIONS

Group	Strain	Sex/Age	No. experiments*	Passage	K_m (NADH), μM $\bar{X} \pm$ SEM
CF	70	M 21 yrs	3	4,5,8	14.5 ± 0.8
	72	F 20 "	2	4,5	13.3 ± 1.4
	46	M 16 "	1	4	12.9
	71	M 7 "	3	4,5,10	15.2 ± 0.9
	69	M 17 "	1	5	10.9
	63	F 3 mos	1	5	12.6
	62	F 7 wks	2	6,13	16.1 ± 0.6
HZ	75	F 35 yrs	1	4	26.3
	76	F 30 "	2	4	22.1 ± 1.7
	78	F 48 "	2	4,5	21.2 ± 0.6
	73	F 54 "	1	5	20.9
	77	M 60 "	1	5	21.3
Control	85	M 6 "	2	4,6	33.9 ± 0.3
	82	F 30 "	2	4,5	32.5 ± 1.8
	84	M 20 "	1	4	33.3
	86	F 49 "	5	5,6,7,9	21.5 ± 2.2
	89	M 58 "	3	5,6	38.7 ± 3.9
	91	M 20 "	3	6,7,10	41.5 ± 1.1
	90	M 20 "	2	6,10	42.8 ± 2.8
	87	F 54 "	2	6,10	40.9 ± 2.5
	93†	F 5 mos	4	5,6,7,12	23.7 ± 0.5
	92	F 3½ mos	3	4,5,8	32.5 ± 1.8
	81	F 32 yrs	2	8,12	31.8 ± 1.5
	94†	F 5 mos	3	3,4	22.4 ± 1.3

* No. separate experiments, each of which was run in duplicate or triplicate.
† Identical twins.

No difference in K_m (NADH) between CF and control for NADH dehydrogenase derived from microsomal (105,000 *g*) fractions was observed (CF: 20.4 ± 3.0 μM [no. = 3], and control: 23.1 ± 2.6 μM [no. = 3]). No difference in apparent K_m of succinate dehydrogenase for $K_3Fe(CN)_6$ was observed (CF: 0.54 ± 0.07 mM [no. = 4], and control: 0.56 ± 0.4 mM [no. = 3]).

DISCUSSION

Michaelis constants (K_m) for mitochondrial NADH dehydrogenase (with the exceptions noted in RESULTS) were clearly different in preparations from cultured fibroblasts of subjects with CF, obligate HZ for CF, and presumed non-HZ controls. The most traditional explanation for K_m differences of an enzyme associated with a Mendelian trait is an altered protein portion of the molecule in question [16, 17]. Other, less likely, explanations for the reported K_m differences among cells with the three genotypes might include: (1) differential post-translational glycosylation of the enzyme; (2) differential effects of mitochondrial inner membrane phospholipid on the binding site of the enzyme; and (3) potential differential effects of mitochondrial Ca or other small molecules on K_m in the three genotypes. Since no evidence exists that NADH dehydrogenase is glycosylated

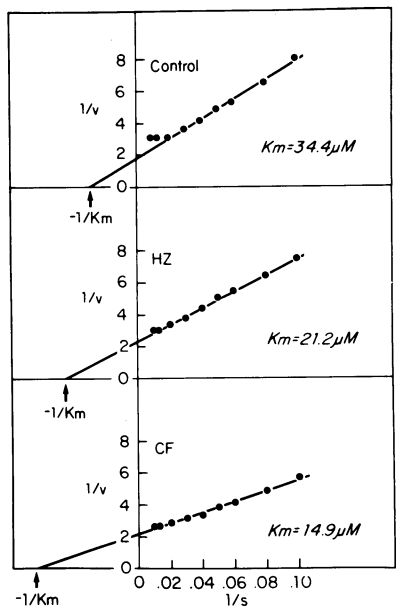

FIG. 1.—K_m (NADH) of NADH dehydrogenase. Representative double reciprocal plots showing the effect of substrate concentration on velocity of NADH oxidation by NADH dehydrogenase. *Each point* is based on triplicate determinations. *Lines* and K_m were calculated by least-squares analysis. Substrate inhibition, most prominent in the control strain shown, was not characteristic of any genotype.

[18], a differential carbohydrate effect on enzyme kinetics in preparations from the three genotypes is unlikely. To examine the possibility of a generalized abnormality of the inner mitochondrial membrane, to which NADH dehydrogenase is tightly bound, we examined kinetics of SDH. SDH is also a flavine-linked dehydrogenase in the mainstream of respiration and electron transport in mitochondria. It is tightly bound to the inner mitochondrial membrane in close proximity to NADH dehydrogenase and also contains iron-sulfur centers. While indirect, and certainly not conclusive, the absence of a difference between CF and control for SDH kinetics would militate against an abnormal phospholipid explanation for the pH optima and kinetic differences found with NADH dehydrogenase. Several bits of evidence rule out a mitochondrial Ca concentration explanation for the K_m differences. During preparation of sample, the use of EDTA or EGTA, discarding of supernatants, and final dilution of about 100 X's of the original lysate would remove any pre-existing Ca concentration differerences in strains with the different genotypes. We examined the effects of a wide range of Ca concentrations in the reaction medium (data not shown). No differential effect of Ca on enzyme activity on preparations from the three genotypes was noted. The preparative steps and dilutions prior to enzyme assays would similarly counter differential effects on K_m of other endogenous small molecules.

More unlikely explanations for the kinetic differences of enzyme obtained from individuals with the three genotypes may exist. The only known and common genetic difference, however, among subjects in the three groups is the presence of two (CF), one (HZ), or probably zero (control) CF alleles. Any secondary, tertiary, or further modification of the enzyme would not likely result in the discrete kinetic differences of NADH dehydrogenase observed among the genotypes.

K_m of NADH dehydrogenase from all known CF individuals was distinct from all HZ and control individuals. K_m from all known HZ individuals was different from nine of the 12 controls. We cannot, of course, know that the three control individuals whose K_m coincides with HZ are indeed carriers for CF. The fact that pH optima of the enzyme from these strains are similar to that from known HZ supports the conclusion that individuals #86, #93, and #94 are HZ. Strains 93 and 94 were obtained from identical twin infants. The finding that the K_m (NADH) of NADH dehydrogenase and pH optimum were similar in preparations from these two individuals enhances the interpretation of the findings.

We have traced increased intracellular Ca in CF to mitochondria and to kinetic differences of mitochondrial NADH dehydrogenase in CF, HZ, and controls. It is hazardous to project from an in vitro situation to intracellular in vivo effects. Nevertheless, the fact that an enzyme complex involved in mitochondrial Ca movement behaves differently from controls in cells from individuals with CF is of interest [19]. The ways in which protein variants associated with mutant genes are identified include such parameters as "different Michaelis constants" and "different pH optima" [17]. We demonstrated that mitochondrial NADH dehydrogenase behaves differently from controls in cells from individuals with CF and obligate carriers for the CF gene in terms of both pH optima and K_m. Greater purification of mitochondrial NADH dehydrogenase is indicated. This is by no means a simple problem: the enzyme is an enormously complex system [18]; fibroblasts are not a rich source of mitochondria; ten 75-cm^2 flasks of cells yield about 5 mg of cell protein. Nevertheless, we are currently attempting to further purify the system from much larger quantities of cultured fibroblasts. These problems aside, data reported here suggest an association between the CF gene and mitochondrial NADH dehydrogenase.

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A preliminary report of this work has been published as an abstract [20].

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