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Assembly of a chimeric respiratory chain from bovine heart submitochondrial particles and cytochrome *bd* terminal oxidase of *Escherichia coli*

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ABSTRACT

Cytochrome *bd* is a terminal quinol oxidase in *Escherichia coli*. Mitochondrial respiration is inhibited at cytochrome bc_1 (complex III) by myxothiazol. Mixing purified cytochrome *bd* oxidase with myxothiazol-inhibited bovine heart submitochondrial particles (SMP) restores up to 50% of the original rotenone-sensitive NADH oxidase and succinate oxidase activities in the absence of exogenous ubiquinone analogs. Complex III bypassed respiration and is saturated at amounts of added cytochrome *bd* similar to that of other natural respiratory components in SMP. The cytochrome *bd* tightly binds to the mitochondrial membrane and operates as an intrinsic component of the chimeric respiratory chain.

1. Introduction

Membranous quinone-reactive proteins are key components of diverse respiratory and photosynthetic electron transfer chains in both prokaryotic and eukaryotic organisms. The quinone/quinol (Q) oxidoreduction at specific quinone-reactive sites (Q-sites) in several cases is coupled with the generation of proton-motive force across the coupling membrane. Extremely hydrophobic natural quinones with 6–10 isoprenoid units also function as a mobile membranous redox pool (buffer) in carrying reducing equivalents between the respiratory chain components [1]. In situ studies on enzymology of quinone oxidoreduction are greatly hampered by quantitative limitation of the endogenous substrate/product. When complete coupled or uncoupled NADH or succinate oxidase activities are measured complexes III and IV operate synchronously with the quinone reductases thus making it difficult, if not impossible to dissect catalytic activities of the individual complexes. Thus, several water-soluble synthetic quinone homologs and/or analogs are commonly used for kinetic assays of individual quinone-reactive complexes [2]. Although this approach is useful, it still suffers from a number of limitations. When low concentrations of a Q-type acceptor/donor are used an enzyme does not operate at its maximal turnover and the true initial rate is hard to detect because of accumulation of the reaction product which may act as an inhibitor. For example, this kinetic behavior has been documented for the succinate:ubiquinone reductase reaction catalyzed by complex II [3,4]. When high concentrations of the water-soluble artificial Q-type acceptors are used as substrate the reaction with other than the natural Q-site associated redox components becomes significant, as exemplified by the loss of rotenone-sensitivity of the NADH:ubiquinone oxidoreductase reaction catalyzed by complex I at high concentrations of Q_1 [5].

Our long-standing interest in the operation of Q-site(s) in mitochondrial membrane-bound NADH and succinate quinone reductases has prompted us to develop reliable assay procedures suitable for studies on steady-state quinone reduction free of those limitations. To achieve this goal we decided to use the purified

Abbreviation: SMP, submitochondrial particles

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detergent solublized cytochrome *bd* quinol oxidase of *Escherichia coli* [6] as a quinone-regenerating system in a continuous coupled NADH:quinone reductase–quinol oxidase assay. We assumed that complex I, in coupled or uncoupled submitochondrial particles and admittedly uncoupled soluble cytochrome *bd* quinol oxidase, will operate independently being kinetically connected by exogenously added water-soluble quinone. An expected advantage of this approach is that in such a system steady-state complex I operation could be measured at any level of quinone reduction that could be reached by unrestricted variation of relative contents of submitochondrial particles (SMP), quinol oxidase, and total concentration of Q_1 in the assay samples. Although this coupled assay was, indeed, operative in the presence of limited amounts of Q_1 and "kinetic excess" of quinol oxidase, we found, unexpectedly, that the system was also operative in the absence of any externally added quinones, thus suggesting that endogenous membrane-bound Q_{10} is accessible to the bacterial quinol oxidase. This report describes some properties of the chimeric respiratory chain assembled from bovine heart SMP and bacterial cytochrome *bd* quinol oxidase.

2. Materials and methods

Inside-out bovine heart SMP were prepared [7], treated with oligomycin, and their NADH and succinate dehydrogenase were activated as described [8]. The cytochrome *bd* terminal quinol oxidase of *E. coli* (strain GO105/pTK1) was prepared as described [9]. The final preparation (2 mg/ml) was dissolved in 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM EDTA and 0.05% *N*-lauroylsarcosine. The heme *d* content (~10 nmol/mg of protein) was estimated from the dithionite-reduced-*minus*-"air-oxidized" spectra assuming $\Delta\varepsilon_{628-607} = 10\,800\ M^{-1}\ cm^{-1}$. All preparations were stored in liquid nitrogen and thawed just before use. The standard reaction mixture was comprised of 0.25 M sucrose, 50 mM Tris–HCl (pH 7.7), and 0.2 mM EDTA. When appropriate, NADH (0.1 mM), potassium succinate (20 mM), Q_1H_2, Q_1 or Q_2, and myxothiazol (0.5 μM) were added to the standard reaction mixture. All assays were performed at 37 °C. The NADH oxidase activity was followed by the absorption decrease at 340 nm assuming $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$. Succinate oxidase activity was followed as an absorption increase at 278 nm (fumarate formation) assuming $\varepsilon_{278} = 0.3\ mM^{-1}\ cm^{-1}$; the latter value was determined by the addition of aliquots of sodium fumarate to the suspension of SMP incubated in the standard reaction mixture in the assay cuvette. The quinol oxidase activity was assayed as an absorption increase at 278 nm with 0.02 mM Q_1H_2 prepared as described [10] assuming $\varepsilon_{278} = 10.2\ mM^{-1}\ cm^{-1}$. Respiration-driven electrogenic activity was estimated as oxonol VI (1.5 μM) response [11] following the absorption change at 624 nm minus 602 nm. Other experimental details are indicated in the legends to the figures and Table 1. We noted that the quinol oxidase activity of cytochrome *bd* with Q_1H_2 as the substrate was low if the reaction was initiated by the addition of the soluble enzyme to the assay mixture (final concentration of *N*-lauroylsarcosine originated from the stock solution in the assay mixture was no more than 1×10^{-4}%). The activity gradually increased upon incubation of the diluted protein and reached a maximum (~10 μmol of Q_1H_2 oxidized per min per mg of protein at 20 μM Q_1H_2) after about 7 min of preincubation. This activation may be related to the similar behavior of cytochrome *bd* from *Azotobacter vinelandii* observed by Jünemann and Rich [12], or more likely, due to a structural rearrangement of the protein caused by detergent dilution. This phenomenon was not further examined, and the quinol oxidase activity assays were measured after 7 min preincubation of the diluted enzyme in the standard assay mixture.

Table 1
Effects of *bd* quinol oxidase on energy transduction by coupled SMP.

		μmol/min per mg of SMP protein[a]		
		− Uncoupler	+ Uncoupler[b]	RCR[c]
	NADH oxidase			
1.	SMP (control)	0.30	1.5	5.0
2.	SMP + QO[d]	0.5	1.3	2.6
3.	As before + 0.5 μM myxothiazol	0.4	0.5	1.2
	NADH:Q_1-reductase[e]			
4.	SMP	0.4	1.0	2.5
5.	SMP + QO[d]	0.5	0.8	1.6

[a] All the activities were measured in the presence of bovine serum albumin (1 mg/ml) in the standard reaction mixture supplemented by 0.1 mM NADH.
[b] Gramicidin D (0.2 μg/ml); respiration rate measured after addition of uncoupler to the same assay sample as depicted in previous column.
[c] Respiratory control ratio.
[d] The assembled system was prepared as described in Fig. 3; the content of *bd* quinol oxidase (QO) added to the reconstitution mixture was 0.15 nmol per mg of SMP protein.
[e] The initial rates of 80 μM Q_1 reduction were measured in the presence of 0.5 μM myxothiazol.

Protein content was determined by the biuret procedure (SMP) or by the Lowry method (quinol oxidase). All fine chemicals were from Sigma–Aldrich (USA).

3. Results

Fig. 1 demonstrates the tracings of NADH oxidation by control (A) and myxothiazol-inhibited (B) SMP. When SMP were treated with excess of the complex III inhibitor myxothiazol their uncoupled NADH and succinate oxidase (not shown) activities decreased to less than 1% of the original (1.1 ± 0.3 and 0.8 ± 0.2 μmol per min per mg of SMP protein, at 37 °C, respectively) activities. If cytochrome *bd* quinol oxidase and a limited amount of the water-soluble ubiquinol homolog, Q_1 were present in the assay mixture (Fig. 1B) the rotenone-sensitive NADH oxidation was restored, as expected.

When myxothiazol-treated SMP were added to the assay mixture containing cytochrome *bd* quinol oxidase of *E. coli* and *no exogenous quinone* their rotenone-sensitive NADH oxidase activity

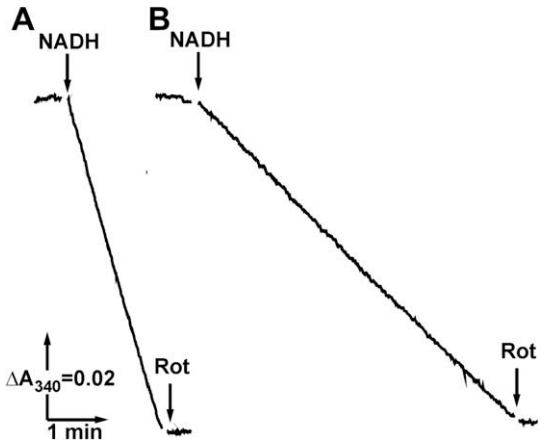

Fig. 1. NADH oxidation by complex III-inhibited submitochondrial particles in the presence of the bacterial quinol oxidase and water-soluble quinone (Q_1). The standard reaction mixture (see Section 2) was supplemented by gramicidin D (0.1 μg/ml). The SMP protein content in all assays was 5 μg/ml; the final concentrations of other addition were: NADH, 0.1 mM; rotenone (Rot), 5 μM; and Q_1, 3 μM. (A) Control, no myxothiazol and Q_1were added and (B) 0.5 μM myxothiazol, 5 μM Q_1, and cytochrome *bd* quinol oxidase (1 μg protein/ml) preincubated in the assay mixture for 7 min were present.