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Underevaluation of Complex I activity by the direct assay of NADH-coenzyme Q reductase in rat liver mitochondria

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Abstract **We have shown that the rate of NADH-coenzyme Q reductase in rat liver mitochondria, assayed using the decyl-ubiquinone analog DB, is underevaluated, probably as a result of its low water solubility. In view of drawbacks encountered using other more soluble acceptors in this system, we demonstrate that the most reliable assay of the physiological rate of CoQ reduction by Complex I is the indirect calculation from the total rate of NADH oxidation and the rate of ubiquinol oxidation, using the pool equation of Kröger and Klingenberg [(1973) Eur. J. Biochem. 34, 358–368].**

Key words: NADH-coenzyme Q reductase; Water solubility; Underevaluation of Complex I activity; Rat liver mitochondrion

1. Introduction

A previous publication from our laboratory [1] pointed out the need for a reliable assay for mitochondrial NADH-coenzyme Q (CoQ) reductase (Complex I; EC 1.6.99.3); in fact this enzyme, that is known to provide half the energy for oxidative phosphorylation in the form of a proton gradient [2], has received novel interest as a consequence of its involvement in some degenerative diseases such as Parkinson's disease, and in the ageing process [3].

The main uncertainty in the assay of Complex I activity is in the choice of a reliable acceptor, since the natural one, CoQ_{10} in most species, cannot be used because of its exceeding insolubility in water: however, in bovine heart mitochondria, we established [1] that CoQ_1, a short chain isoprenoid homolog of the physiological acceptor, elicits high enzymatic rates, presumably comparable to those existing in the natural respiratory chain. Other widely used quinone homologs, such as CoQ_0, CoQ_2, and analogs, as decyl-ubiquinone (DB), have consistent drawbacks preventing attainment of optimal rates when used as substrates of the enzyme [1].

That finding in beef heart mitochondria, however, may not have to be necessarily applicable to other systems. A meaningful assay for Complex I requires the reaction to be sensitive to rotenone, a specific inhibitor of Complex I acting at or near the natural quinone-binding site(s) [4]; in bovine heart mitochondria CoQ_1 reduction is over 90% rotenone-sensitive, indicating that CoQ_1 is reduced at the physiological acceptor site. However, in a study on Complex I activity in Leber's optical neuropathy [5], CoQ_1 could not be employed because its reduction was substantially insensitive to rotenone; in its place, a saturated chain undecyl analog was employed with satisfactory results.

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Subsequent observations in our laboratory using rat liver mitochondria have also shown an unfitness of CoQ_1 as the electron acceptor, for the identical reason as above that its reduction is largely rotenone insensitive; the same studies have also shown that this inconvenience is not encountered using decyl-ubiquinone (DB), a commercially available saturated chain analog which is widely employed in bioenergetics in both its oxidized and reduced form.

In this report, however, we show that NADH-CoQ reductase activity calculated from the direct assay using DB as acceptor is significantly underevaluated when compared with the activity indirectly calculated from the total observed rate of NADH oxidation and the rate of ubiquinol oxidation by means of the 'pool equation' of Kröger and Klingenberg [6].

2. Materials and methods

All chemicals, including DB, were purchased from Sigma Co., St. Louis, MO. When necessary, the DB was reduced according to Rieske [7]. CoQ_1 was a gift from Eisai Co., Tokyo.

Submitochondrial particles (SMP) were prepared by sonication of beef heart mitochondria [1].

Liver mitochondria were prepared with the procedure of Kun et al. [8] from six male albino rats of the Wistar strain, aged 6 months, purchased from Charles River Italia SpA, Milano.

Enzymatic activities were assayed at 30°C, essentially as described in [9], after one cycle of freezing and thawing of the isolated mitochondria, which was found to completely remove the permeability barrier to NADH without significantly damaging the enzyme. Protein was determined with a biuret method [10] with addition of 10% Na-deoxycholate.

Ubiquinol cytochrome *c* reductase and cytochrome oxidase were assayed in a 25 mM K-phosphate buffer, pH 7.5, with 1 mM KCN in the reductase assay, and using 48 μM ferricytochrome *c* and 9.8 μM decyl-ubiquinol (DBH_2) in the reductase and 40 μM ferrocytochrome *c* in the oxidase assay. The activities were evaluated by monitoring the absorbance change of cytochrome *c*, respectively upon reduction or oxidation, at 550 minus 540 nm in a Sigma Biochem ZWS 2 dual wavelength spectrophotometer equipped with a rapid mixing apparatus, and using an extinction coefficient of 19.1 $mM^{-1} \cdot cm^{-1}$.

Aerobic ubiquinol-2 oxidation was measured in the same assay as ubiquinol cytochrome *c* reductase, but following the increase of absorbance at 275 nm due to oxidized quinone using an extinction coefficient of 12.5 $mM^{-1} \cdot cm^{-1}$.

Aerobic NADH oxidation was assayed in a 50 mM KCl, 10 mM Tris-HCl, 1 mM EDTA buffer, pH 7.4, using 75 μM NADH and 40 μg/ml of mitochondrial protein and following the decrease of absorbance of NADH at 340 minus 380 nm using an extinction coefficient of 5.5 $mM^{-1} \cdot cm^{-1}$. NADH-DB reductase was assayed in the same system except for including in the assay medium 1 mM KCN and 1 μM antimycin A, to completely block aerobic oxidation, and 60 μM DB as the electron acceptor.

3. Results and discussion

It is unanimously accepted after the convincing evidence of Kröger and Klingenberg [6] and widely confirmed thereafter

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[11], that NADH oxidation occurs through a mobile homogeneous CoQ pool which connects Complex I with the bc_1 Complex; being the CoQ/CoQH$_2$ redox couple the common intermediate between the two enzyme activities, then the rate of NADH-CoQ reductase (V_{red}) would be significantly *higher* than the rate of either NADH cytochrome *c* reductase or of NADH oxidase (V_{obs}); in fact it would approach the latter only for a large rate excess of the portion of the chain that reoxidizes ubiquinone (V_{ox}), as it readily appears from the pool equation [6]:

$$V_{obs} = V_{ox} \times V_{red}/(V_{ox}+V_{red})$$

The reduction of a CoQ analog, as CoQ_1, that is exploited to assay Complex I activity, is considered to reflect the reduction of the physiological endogenous CoQ pool (CoQ_{10} in the mitochondria from most mammals, largely CoQ_9 in rodents).

In bovine heart SMP the rate of CoQ_1 reduction by NADH is high and over 90% sensitive to the specific inhibitors of Complex I [1], the best known of which is rotenone [4].

In order to test whether the rate of CoQ_1 reduction quantitatively reflects the rate of reduction of endogenous CoQ, aerobic NADH and ubiquinol oxidations were also determined and taken as V_{obs} and V_{ox}, respectively, in the pool equation, to calculate the rate of NADH-CoQ reductase (V_{red}) with the endogenous pool.

Table 1 shows that V_{red}, thus determined, very closely agrees with the experimentally determined rate of NADH-CoQ_1 reductase. The rate of CoQ_1 reduction in bovine SMP is therefore a quantitative representation of the rate of reduction of the physiological CoQ pool. We have found out, however, that this is not the case in rat liver mitochondria.

Preliminary experiments showed that CoQ_1 was reduced by NADH in rat liver mitochondria at rates comparable with those elicited by molecular oxygen; it was, however, apparent that CoQ_1 reduction was almost completely insensitive to rotenone (the inhibition ranged from zero to 30%). On the other hand DB reduction was around 90% inibited by rotenone, in agreement with that of molecular oxygen. This means that DB reduction occurs at or near the physiological acceptor site, as outlined above.

Inspection of Table 2, exhibiting the mean of the enzyme determinations on six different preparations of rat liver mitochondria, reveals an incongruency between the rates of NADH oxidation by molecular oxygen and by the CoQ analog DB; in fact NADH oxidation by DB is slightly slower than NADH oxidation by oxygen in spite of having used the same assay conditions. It has to be clearly enphasized that, as a consequence of V_{ox} and V_{red} being of the same order of magnitude [12], the latter should be *much higher* than V_{obs}, as a mere kinetic result of the pool behavior [6].

Table 1
Enzymatic activities of beef heart SMP and calculation of NADH-CoQ reductase activity from the pool equation [6]

Enzyme	Specific activity[a] (μmol·min^{-1}·mg^{-1})
NADH-O_2	0.83
Ubiquinol-O_2	2.45
NADH-CoQ_1	1.19
NADH-CoQ (V_{red})	1.25

[a] Activities of ubiquinol-O_2 and NADH-CoQ_1 are V_{max} obtained by ubiquinol-2 and CoQ_1 titrations, respectively. Table 1 reports a representative example among determinations in different batches of SMP.

Table 2
Enzymatic activities of rat liver mitochondria and calculation of NADH-CoQ reductase activity from the pool equation [6]

Enzyme	Specific activity[a] (μmol·min^{-1}·mg^{-1})	
NADH-O_2	0.157 ± 0.037	
Ubiquinol-Cyt. *c*	1.099 ± 0.373	
Cyt. *c*-O_2	1.263 ± 0.348	
NADH-DB	0.148 ± 0.018	
NADH-CoQ (V_{red})	0.226 ± 0.065	$P<0.01$[b]

[a] Mean ± standard deviation from 6 different preparations.
[b] Statistically significant with respect to direct assay of NADH-DB reductase.

In view of the above difficulty, we have recalculated the rate of Complex I activity (V_{red}) by the appropriate rearrangement of the pool equation, as we had previously done in the bovine SMP.

In the rat liver experiments, NADH oxidation by oxygen was taken as V_{obs}; since aerobic ubiquinol oxidation in this system was not reproducible, the V_{ox} was considered equal to the rate of ubiquinol-cytochrome *c* reductase normalized for 2-electron transfer by dividing the rate of cytochrome *c* reduction (a 1-electron carrier) by two. This assumption was validated by the fact that ubiquinol cytochrome c reductase is the rate-limiting step in V_{ox} in our experimental conditions, as demonstrated by Table 2 showing that cytochrome oxidase activity is slightly higher than ubiquinol-cytochrome *c* reductase.

By using the pool equation we have recalculated the activity of NADH-CoQ reductase and obtained the values also shown in Table 2, significantly higher than those obtained by direct assay of NADH-DB reductase.

The rationale of our calculation is independent of the CoQ concentration in the membrane. However, if CoQ concentration in the inner membrane of rat liver mitochondria is not saturating for maximal rate of electron transfer, as we have found is the case in beef heart mitochondria [13], the rate of NADH oxidation through the CoQ pool would not be maximal. In such case, application of the pool equation using $V_{obs(max)}$ would increase the rate of NADH-CoQ reductase even further over the values obtained by direct assay.

The lack of a quantitative assay for NADH-CoQ reductase is limited to those mitochondria in which reduction of CoQ_1, which was found to be the most reliable electron acceptor in beef heart mitochondria [1], has a large rotenone-insensitive component. The reason why CoQ_1, contrary to DB, is reduced by an additional rotenone-insensitive pathway may be linked to a selectivity of the rotenone-insensitive NADH dehydrogenase(s) for isoprenoid quinones against the straight chain analogs.

The nature of such rotenone-insensitive dehydrogenase(s) in liver mitochondria is probably to be identified in the high NADH-cytochrome b_5 (or *c*) reductase of the outer membrane [14]; we cannot, however, dismiss the possibility that part of the rotenone-insensitive CoQ_1 reductase activity belongs to Complex I itself, since it was found even in bovine heart mitochondria at high quinone concentrations [15]; it may be speculated that the non-physiological sites are significantly more available or active in liver mitochondria than in heart mitochondria.

The results of this study caution investigators of Complex I activity to use the suitable electron acceptor selected through a choice dictated by appropriate experimentation. When rates approaching as closely as possible the physiological rates are required, such as for comparative purposes between experimental groups, it would be advisable to calculate the CoQ reductase activity indirectly from the pool equation, as performed in this study.

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