The intramitochondrial ATP/ADP-ratio controls cytochrome *c* oxidase activity allosterically

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Abstract Recently the signal transduction function for oxidative phosphorylation was found to be second order in ADP [Jeneson, J.A.L., Wiseman, R.W., Westerhoff, H.V. and Kushmerick, M.J. (1996) J. Biol. Chem. 271, 27995–27998], but the molecular mechanism of signal transduction remained unclear. Previously we described inhibition of cytochrome *c* oxidase by intramitochondrial ATP, accompanied by a change of hyperbolic into sigmoidal kinetics. The present study describes a sigmoidal relationship also between the ascorbate respiration of reconstituted cytochrome *c* oxidase and intraliposomal ADP concentration. Its possible role in the control of oxidative phosphorylation and cell respiration is discussed.

Key words: Cytochrome *c* oxidase; Respiratory control; Nucleotide binding; Allosteric effector; ATP/ADP ratio

1. Introduction

'Respiratory control' was originally described as the stimulation of the respiration of isolated mitochondria by ADP [1,2]. With the advent of Peter Mitchell's chemiosmotic hypothesis [3] this phenomenon could be explained by the proton motive force Δp across the inner mitochondrial membrane, consisting of an electrochemical potential and a pH gradient ($\Delta p = \Delta\Psi - 61.5\ \Delta pH$). Δp is produced by the three proton pumps of the respiratory chain (NADH dehydrogenase, cytochrome *c* reductase, and cytochrome *c* oxidase), and its energy is used by the proton gradient-driven ATP synthase for the synthesis of ATP. The electron transfer activity of the proton pumps is controlled by Δp. It is assumed that uptake of ADP into mitochondria stimulates the ATP synthase, accompanied by decreased Δp, consisting mainly of $\Delta\Psi$, which in consequence stimulates the activity of the proton pumps [4]. Chance et al. [5] considered ADP to be the principal control parameter of oxidative metabolism in skeletal muscle, and assumed further control by NADH and the O_2 delivery. In perfused hearts, however, neither the mitochondrial membrane potential [6] nor the cytosolic ADP level [7], calculated from ^{31}P-NMR data [8], correlated with the rate of respiration. Thus it is still unclear what ultimately limits myocardial performance under awake exercising conditions [9].

Jeneson et al. [10] observed in human skeletal muscle in vivo a sigmoidal relationship between the rate of oxidative phosphorylation, which corresponds to the rate of cell respiration, and the concentration of cytosolic ADP. The signal transduction function for oxidative phosphorylation was suggested to be second order in ADP. The sigmoidal relationship was assumed to be based on allosteric instead of Michaelis-Menten kinetics of the adenine nucleotide translocation in mitochondria. This interpretation, however, was questioned recently by Portman et al. [11]. Previously we have shown that the well known hyperbolic kinetics of cytochrome *c* oxidase, measured also in the presence of ADP, is changed into sigmoidal kinetics in the presence of ATP [12], due to the exchange of bound ADP by ATP at the matrix domain of subunit IV [13,14]. ATP acts as an allosteric inhibitor, leading to full inhibition of activity at low concentrations of cytochrome *c* (< 2–4 μM). In the present study a sigmoidal relationship was also found between the ascorbate respiration of reconstituted cytochrome *c* oxidase and the intraliposomal ADP concentration, when [ADP+ATP]=constant. This relationship is independent of Δp and offers a molecular explanation for the sigmoidal relationship between the rate of oxidative phosphorylation and cytosolic ADP concentration, as observed in vivo [10].

2. Materials and methods

Cytochrome *c* oxidase was isolated from bovine heart mitochondria as previously described [15]. The enzyme was reconstituted into liposomes consisting of asolectin (L-α-phosphatidylcholine from soybean, type II-S from Sigma) and 5% cardiolipin (Sigma) in 10 mM K-HEPES, pH 7.4, 40 mM KCl, 1.5% sodium cholate in the presence of 5 mM [ATP+ADP] at the indicated ratio by the hydrophobic adsorption method [16] applying Amberlite XAD-2 (Serva, Heidelberg), followed by dialysis against 10 mM K-HEPES, pH 7.4, 40 mM KCl. The ascorbate respiration of proteoliposomes (75 nM heme aa_3) was measured polarographically in 10 mM K-HEPES, pH 7.4, 40 mM KCl, 18 mM ascorbate at increasing concentrations of cytochrome *c* (0–60 μM). When indicated, the uncouplers valinomycin (1 μM) and CCCP (3 μM) were added. The rate of ascorbate respiration is presented as turnover number (TN = mol 1/4 $O_2 \times$ mol heme aa_3^{-1} s^{-1}).

3. Results

In a previous study the allosteric inhibition of cytochrome *c* oxidase, via exchanging tightly bound ADP by ATP at the matrix domain of subunit IV, was shown [12]. The Michaelis-Menten type of cytochrome *c* oxidase kinetics, measured in the presence of ADP, changed into a sigmoidal activity/cytochrome *c* concentration relationship in the presence of intra-

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Abbreviations: PEP, phosphoenolpyruvate; PK, pyruvate kinase; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; $\Delta\Psi$, mitochondrial membrane potential; Δp, proton motive force

This paper is dedicated to Professor Britton Chance, the pioneer in the research on mitochondrial respiration.

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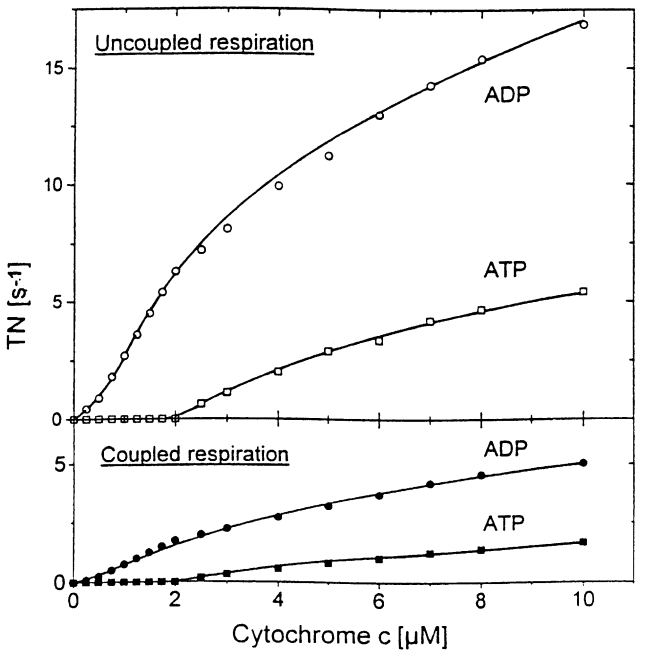

Fig. 1. Cytochrome *c* oxidase activity is allosterically inhibited by ATP, independently of a proton motive force. Presented is the ascorbate respiration of cytochrome *c* oxidase from bovine heart, reconstituted into liposomes consisting of asolectin and 5% cardiolipin in the presence of 5 mM ADP (circles) or 5 mM ATP (squares). The rate of ascorbate respiration (TN = mol 1/4 O_2 × mol heme aa_3^{-1} s^{-1}) was measured polarographically at increasing concentrations of cytochrome *c* in the absence (coupled respiration, closed symbols) or presence of CCCP and valinomycin (uncoupled respiration, open symbols).

liposomal (or intramitochondrial) ATP. The maximal Hill coefficient n_H, calculated from the ascorbate respiration of Tween-80-solubilized mitochondria in the presence of ATP and an ATP regenerating system (PEP and PK), as well as from the activity of reconstituted cytochrome *c* oxidase from bovine heart in the presence of ATP, was 2.04 and 1.97, respectively, suggesting cooperative interaction of two cytochrome *c* binding sites, which are probably located each at one monomer of the dimeric enzyme complex [17].

Fig. 1 shows the influence of the proton motive force Δp on the allosteric inhibition of cytochrome *c* oxidase by ATP. Cytochrome *c* oxidase was reconstituted into liposomes in the presence of 5% cardiolipin and either 5 mM ATP or 5 mM ADP. The extraliposomal nucleotides were removed by dialysis. The ascorbate respiration was measured in the absence (coupled respiration) or presence of valinomycin and CCCP (uncoupled respiration). Under both conditions, differing in respiration rates by a factor of 3, the percentage of allosteric ATP inhibition was the same, indicating its independence of the proton motive force across the membrane.

To determine the ATP/ADP ratio at which half-maximal change of hyperbolic into sigmoidal kinetics occur, we measured the kinetics of ascorbate respiration of the reconstituted enzyme from bovine heart at increasing cytochrome *c* concentrations in the presence of different intraliposomal ATP/ADP ratios. The resulting values of the Hill coefficient increased from $n_H = 1.09$ at 0% ATP (5 mM ADP) to $n_H = 1.97$ at 100% intraliposomal ATP (5 mM) in the presence of the ATP regenerating system (PEP+PK), as presented in Table 1. Half-maximal stimulation of respiration, measured in the presence of 10 μM cytochrome *c*, was obtained at about 0.17 mM intraliposomal ADP (96.6% ATP, or at [ATP]/[ADP] = 28).

The data of ascorbate respiration at different cytochrome *c* concentrations of the experiment described in Table 1 were plotted against increasing concentrations of intraliposomal ADP, where [ATP+ADP] = 5 mM, as shown in Fig. 2. For all concentrations of cytochrome *c* sigmoidal curves were obtained. The fitted solid curves for each concentration of cytochrome *c* were based on functions of the computer program Origin 5.0. The graphically determined Hill coefficients of the curves varied, and ranged from $n_H = 2.12$ to 3.96 with an average of $n_H = 2.86$.

4. Discussion

The results of this study indicate a control of cytochrome *c* oxidase activity by the intramitochondrial ADP concentration (or ATP/ADP ratio), which is independent of the proton motive force across the membrane. ADP stimulates (and ATP inhibits) cytochrome *c* oxidase activity via binding to a high-affinity binding site for ATP or ADP at subunit IV [12–14]. The sigmoidal relationship between cytochrome *c* oxidase activity and intraliposomal [ADP] corresponds to that described by Jeneson et al. [10] for the dependence of the rate of oxidative phosphorylation, corresponding to the rate of cell

Table 1
The Hill coefficient n_H of the ascorbate respiration of reconstituted cytochrome *c* oxidase, measured at increasing concentrations of cytochrome *c*, changes from one to two with increasing intraliposomal ATP/ADP ratios

% ATP	mM [ADP]	TN at 10 μM cytochrome *c*	n_H
0	5	30.0	1.09
70	1.5	30.0	1.09
80	1.0	30.0	1.09
90	0.5	29.3	1.29
95	0.25	25.7	1.39
96	0.2	22.9	1.41
97	0.15	16.7	1.68
98	0.1	13.0	1.81
99	0.05	12.7	1.88
100	0	12.5	1.87
100 (+PEP+PK)	0	12.5	1.97

Cytochrome *c* oxidase from bovine heart was reconstituted into liposomes in the presence of 5% cardiolipin and the indicated concentrations of ADP and ATP ([ATP+ADP] = 5 mM), followed by dialysis. The ascorbate respiration of proteoliposomes was measured polarographically at increasing concentrations of cytochrome *c* (see legend to Fig. 2). The Hill coefficient n_H was determined graphically from titration curves as previously described [12].

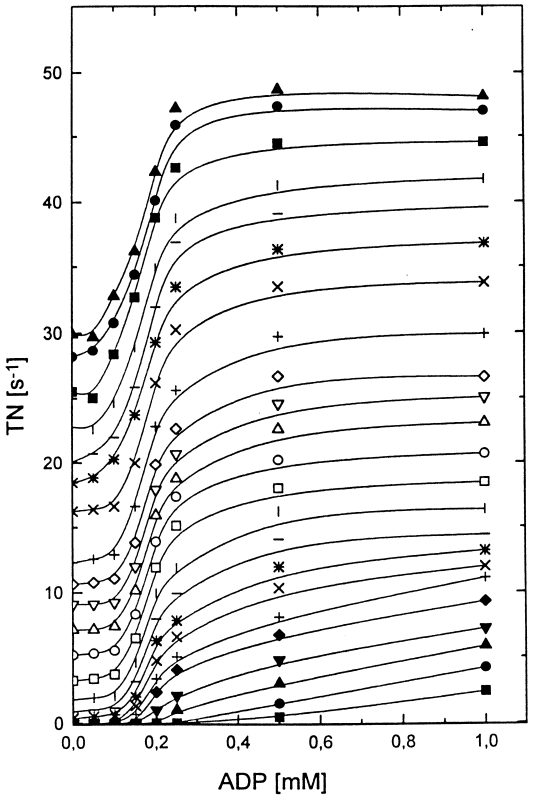

Fig. 2. Control of cytochrome *c* oxidase activity by ADP or the ATP/ADP ratio. Cytochrome *c* oxidase from bovine heart was reconstituted into liposomes in the presence of 5% (w/v) cardiolipin and the indicated concentrations of ADP and ATP ([ATP+ADP] = 5 mM), followed by dialysis. The ascorbate respiration of the different proteoliposomes was measured polarographically at the following concentrations of cytochrome *c* (in μM from bottom to top, indicated by different symbols): 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3,0, 4.0, 5.0, 6.0, 7.0, 8.0, 10, 15, 20, 25, 30, 40, 50, 60. The solid lines were fitted with a computer program (Origin 5.0).

respiration, on cytosolic ADP concentration, determined in the human forearm flexor muscle by ^{31}P-NMR spectroscopy. Their calculated Hill coefficients were $n_H = 2.11$ for in vivo measurements and $n_H = 2.1$–2.9 with isolated mitochondria. At different cytochrome *c* concentrations we found values between $n_H = 2.12$ and 3.96 (average 2.86), indicating similar Hill coefficients for the dependence of respiration on [ADP] under in vivo conditions, with isolated mitochondria, as well as with purified and reconstituted cytochrome *c* oxidase.

In the study of Jeneson et al. half-maximal stimulation of oxidative phosphorylation was found at 50 μM ADP, whereas in our study about 170 μM ADP induced half-maximal stimulation of ascorbate respiration with the reconstituted enzyme, when [ATP+ADP] = 5 mM. While with ^{31}P-NMR spectroscopy the cytosolic [ADP] is determined, in our study the influence of intramitochondrial [ADP] was investigated. Due to the mitochondrial membrane potential and the electrogenic nature of the ADP/ATP carrier, cytosolic ATP/ADP ratios are higher than matrix ATP/ADP ratios. Correspondingly, free [ADP] is expected to be higher in the mitochondrial matrix than in the cytosol. Furthermore, the total intramitochondrial concentration of [ADP+ATP] in heart mitochondria could be higher than 5 mM, as applied in our study, since in liver mitochondria a concentration of 12–16 mM [ADP+ATP] was found in vivo [18].

Although cytochrome *c* oxidase represents the irreversible step of the respiratory chain, its activity is not assumed to represent the rate-limiting step of cell respiration. From application of the metabolic control analysis [19], a manifold excess of cytochrome *c* oxidase capacity over the amount required to support the endogenous respiration of isolated mitochondria was concluded [20–22]. A limitation of metabolic control analysis applied to isolated mitochondria, as compared to the in vivo situation, however, is the significant alteration of the situation due to the possible loss of essential metabolites and to the absence of the cytosolic substrate and coenzyme environment. Recent metabolic control analysis of respiration in intact cultured cells revealed a tight control of cell respiration by cytochrome *c* oxidase, exceeding its capacity above the endogenous respiratory activity by only 25% [23]. In saponin-permeabilized muscle fibers a higher control strength for cytochrome *c* oxidase was found, in particular at lower oxygen pressure [24].

It is generally assumed that the control of mitochondrial respiration (or oxidative phosphorylation) by ADP involves Δp as a variable parameter in the signal transduction pathway [4]. According to this view, Δp must decrease for stimulating the rate of oxidative phosphorylation. In perfused hearts, however, little influence of the rate of cell respiration on Δp at the inner mitochondrial membrane was measured [6]. Since the allosteric control of cytochrome *c* oxidase by intramitochondrial [ADP] is independent of Δp (see Fig. 1), its participation in the control of cell respiration according to the utilization of ATP would contribute to the homeostasis of Δp in vivo. The control of cytochrome *c* oxidase by intramitochondrial [ADP] or the ATP/ADP ratio, however, is under further control of thyroid hormones. 3,5-Diiodothyronine was shown to completely abolish the allosteric inhibition of cytochrome *c* oxidase by ATP, via specific binding to subunit Va [25]. Subunit Va is located on the matrix side, adjacent to the ADP or ATP binding matrix domain of the transmembranous subunit IV [17]. Interestingly, in bacteria, lacking the corresponding eukaryotic subunit IV, the control of cytochrome *c* oxidase by nucleotides appears to be absent [26]. Although the described allosteric control of cytochrome *c* oxidase activity could represent the pacemaker of cell respiration, other steps in the mitochondrial respiratory chain (e.g. NADH dehydrogenase and cytochrome *c* reductase) could as well be controlled by the matrix ATP/ADP ratio which, however, remains to be investigated.

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