Detection of the local H^+ gradients on the internal mitochondrial membrane

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Abstract Respiration-dependent responses of a pH probe (fluorescein isothiocyanate, FITC), covalently bound to the membrane proteins of mitochondria and submitochondrial particles (SMP) have been studied. A spectral shift indicating FITC deprotonation was observed when respiration was activated in coupled mitochondria. Such a response was increased by valinomycin and reduced by uncoupler. Some FITC deprotonation was detected in the presence of excess of an uncoupler, but the response was smaller and insensitive to valinomycin. FITC deprotonation was also observed in submitochondrial particles after succinate addition. In this case it was not affected by uncoupler. Increase in the buffer concentration was found to (i) decrease the FITC response and (ii) increase the rate of uncoupled respiration in both mitochondria and submitochondrial particles. The results are consistent with the assumption that respiration initiates appearance of local H^+ activity gradients on the inner side of the internal mitochondrial membrane during the steady-state H^+ pumping. We suggest that the formation of this gradient is due to kinetic barrier to proton transfer from the bulk phase to the respiratory proton pump vicinity.

Key words: Mitochondrion; Submitochondrial particle; Fluorescein isothiocyanate; Respiratory proton pump; Local H^+ activity gradient

1. Introduction

Existence of the local coupling of electron transport and phosphorylation in mitochondria and chloroplasts is not firmly proved yet. This is due to lack of direct experiments testing this hypothesis. Recent data obtained for several simple systems [1–4] indicate that the local coupling mechanism seems to be possible.

Membrane proteins can be considered as immobilized buffers [5]. Proton transfer in such systems is relatively slow [5,6]. In accordance with the local electroneutrality principle, it requires H^+/Me^+ exchange and is usually associated with slow volume and conformation changes [6,7]. These properties allow us to consider the membrane protein-water interphase in mitochondria and submitochondrial particles (SMP) as a specific compartment where local H^+ activity gradients may exist. We tried to detect such gradients using the pH probe fluorescein isothiocyanate (FITC), covalently bound to mitochondrial proteins. We managed to detect FITC deprotonation in response to succinate addition in the uncoupled mitochondria at low K^+ concentration. This effect is insensitive to valinomycin addition as well as to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) excess. The same phenomenon was found in the inside-out SMP. The results are discussed in terms of 'mosaic protonic coupling' model [8,9].

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Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; SMP, submitochondrial particles; TTFA, tenoyltrifluoroacetone; Tris, tris(hydroxymethyl)-aminomethane

2. Materials and methods

Bovine heart mitochondria were isolated as described elsewhere [10]. Coupled inside-out SMP were prepared as described by Vinogradov et al. [11]. ATPase activity of such SMP is insensitive to atractyloside [12]. The uncoupled respiration of FITC-labeled SMP with succinate as a substrate was insensitive to polylysine (up to concentrations of 6 μg/ml) and cytochrome *c*. Succinate dehydrogenase activity was determined as described by Burbaev et al. [13].

Rat liver mitochondria were prepared as described in [14]. Labelling mitochondria with FITC was performed as described earlier [15].

The SMP suspension (0.5 ml, 10 mg protein) was labelled with dimethylsulfoxide (DMSO) FITC solution (final concentration 3×10^{-4} M) in the medium (a) containing 0.23 M sucrose, 10 mM KCl, 0.5 mM ethylenediaminetetraacetate (EDTA), 2.5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-HCl, 2.5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH, pH 6.7. The suspension was stirred for 10 min at 0°C. In order to remove the excess of monovalent cations mitochondrial (SMP) suspension was incubated with 70 nM nigericin for 5 min. The excess of FITC was removed by bovine serum albumin (BSA) (10 mg dissolved in 1 ml of the same medium). The suspension was incubated for 2 min at 0°C and then diluted with 3 ml of the labelling medium (a). SMP were sedimentated by centrifugation at $105\,000\times g$ for 30 min. This procedure allows to remove more than 99% of unbound FITC. All the preparation takes about 1 h.

While varying the buffer capacity in experiments below (Figs. 2–4) ionic strength and osmolarity of the media were kept constant choosing appropriate KCl (1–10 mM) and sucrose concentration. The samples were prepared separately in the media with high and low buffer capacity with no effect on probe binding.

In the experiments with SMP the medium with low buffer capacity was the same as described above (medium (a)) and the medium with high buffer capacity (b) containing 0.24 M sucrose, 0.25 mM EDTA, 15 mM HEPES-HCl, 15 mM MES-KOH, pH 6.7 (b). For mitochondria the media with low (c) and high (d) buffer capacity were the following: 0.24 M sucrose, 4.8 mM KCl, 0.25 mM EDTA, 1.5 mM HEPES, pH 7.5 (c); 0.23 M sucrose, 0.25 mM EDTA, 15 mM HEPES, pH 7.5 (d).

All measurements were carried out in the same media in which the samples were prepared.

The differential absorption spectra of the FITC-labeled SMP and mitochondria were recorded with a Hitachi-557 spectrophotometer within the wavelength range 400–600 nm, recording time approx. 1.5 min.

The amount of bound FITC, determined spectrophotometrically, was 10 nmol/mg protein. The effective p*K* value of the bound probe was 6.7.

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Oxygen consumption was measured with a Clarke electrode.

The chemicals used were: succinic acid, tris(hydroxymethyl)aminomethane (Tris), HEPES, MES, FCCP, nigericin, valinomycin, oligomycin (Sigma); EDTA, rotenone (Serva); fluorescein isothiocyanate, BSA (fatty-acid free), KCl (Fluka); DMSO (Aldrich).

3. Results and discussion

Fig. 1A shows the amount of bound FITC depending on the total FITC amount added to the suspension while labelling mitochondria. At 10–15 nm FITC/mg protein saturation occurs indicating that the number of binding sites is not very high. The value of probe responses mentioned below was proportional to the amount of bound probe (Fig. 1B).

As was shown in the previous paper [15], the probe penetrates through the inner membrane and thus attaches to the proteins located on both sides. The pH increase in matrix caused by respiration activation in coupled mitochondria leads to deprotonation of the internal fraction of FITC. This effect is increased by valinomycin and reduced by FCCP (Fig. 2B).

A similar FITC response to succinate addition was observed in uncoupled mitochondria (Fig. 2A). The increase in absorption (at 503 nm) reflects the dye deprotonation (Fig. 2A, spectrum 1). Unlike coupled mitochondria, in this case neither the excess of FCCP nor valinomycin affect the probe response markedly (Fig. 2B). The inhibitor of respiration tenoyltrifluoroacetone (TTFA) reduces deprotonation caused by succinate (Fig. 2A, spectra 1, 3). The response of the probe is much lower in an uncoupled system than in a coupled one (Fig. 2B).

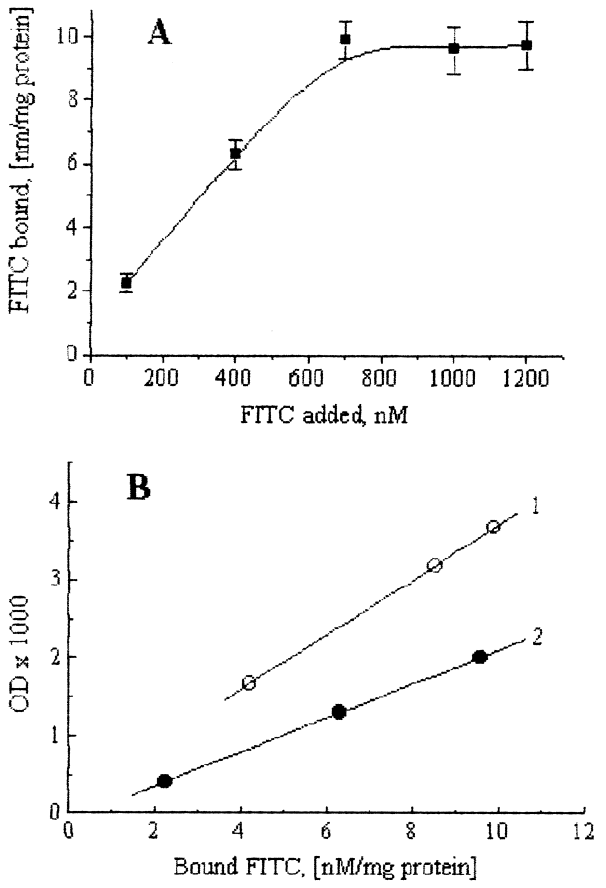

Fig. 1. Mitochondria with various amounts of bound FITC. A: Amount of bound FITC depending on the total added amount. B: The absorption increase at 503 nm after succinate addition depending on the bound FITC amount in uncoupled mitochondria. 1: Incubation medium with low buffer concentration: 0.24 M sucrose, 4.2 mM KCl, 0.25 mM EDTA, 1.5 mM Tris-HCl, pH 7.5. 2: Incubation medium with high buffer concentration: 0.23 M sucrose, 0.25 mM EDTA, 15 mM Tris-HCl, pH 7.5.

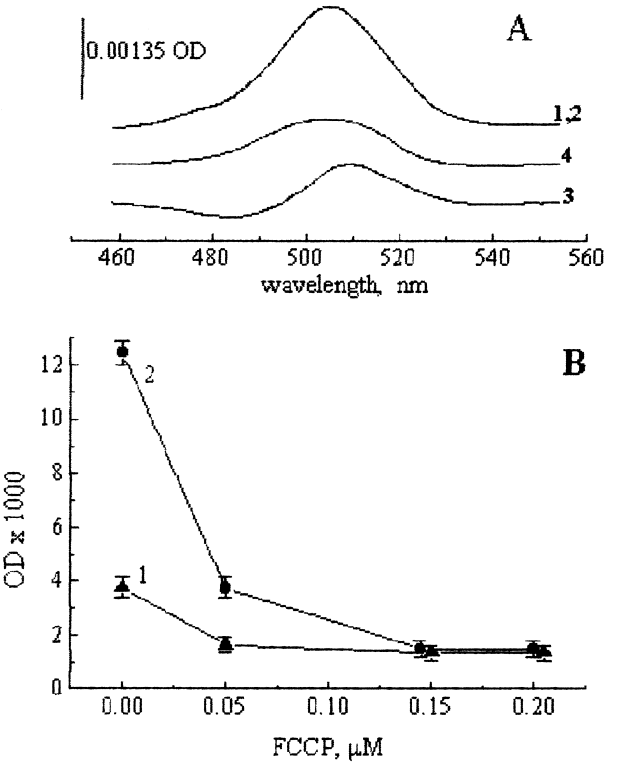

Fig. 2. Bound FITC responses to respiration activation in mitochondria. A: The differential spectra which are observed after subsequent additions: (1) succinate; (2) valinomycin, 2 nM; (3) TTFA, 5 μM; incubation medium (c); (4) succinate, incubation medium (d). Mitochondria previously uncoupled by 100 nM FCCP. B: The influence of previously added FCCP (1) and following valinomycin (2 nM) addition (2) on the value of FITC response to succinate addition (ΔA_{503}); incubation medium contains 0.24 M sucrose, 4.2 mM KCl, 0.25 mM EDTA, 1.5 mM Tris-HCl, pH 7.5.

It was found that the higher pH-buffer concentration is in the incubation medium, the lower is FITC response (Fig. 2A, spectra 1, 4). This effect does not depend on the buffer chemical structure. Similar results were obtained with Tris (Fig. 1B, 1, 2) or HEPES (Fig. 2A, spectra 1, 4). On the other hand, in media with the same buffer capacity but different ionic strength, responses of bound FITC are higher in the media with higher ionic strength [15]. These facts indicate that pH buffer has a specific influence on FITC response in uncoupled mitochondria. This allows to assume that the main factor affecting probe protonation/deprotonation is the local H^+ activity in membrane protein-water interphase, but not the conformational changes in membrane proteins induced by respiration.

The results are consistent with the assumption that the buffers used penetrate through the mitochondrial membrane. It is noteworthy that FITC treatment increases permeability of the membrane to monovalent ions [15].

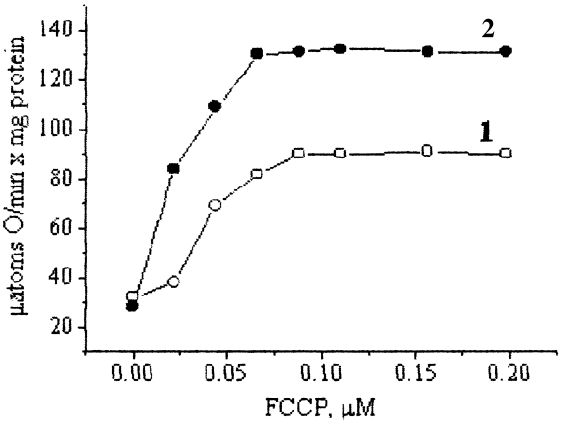

Fig. 3. The influence of buffer capacity on the mitochondrial respiration rates at various uncoupler concentrations. 1: Incubation medium (c), with low buffer concentration. 2: Incubation medium (d), with high buffer concentration.

As the respiration activation is followed by the FITC response, reflecting H^+ activity decline, the responding probe fraction should be located on the inner side of the internal mitochondrial membrane. We suggest that this H^+ activity decline is due to a kinetic barrier for the H^+ transfer from matrix to membrane proteins. This suggestion is supported by slow kinetics of the FITC response. It reaches a maximum in 2–3 min after succinate addition, and dissipates in 10–20 min after respiration was restrained by an inhibitor (TTFA or myxothiazol). The role of this barrier is influenced by buffer concentration: at high buffer concentrations the local H^+ activity gradient is lower (Fig. 2A), whereas the uncoupled respiration is faster (Fig. 3).

The presence of a similar barrier was reported earlier [16,17] for H^+ transfer through the gramicidin channel inserted in BLM.

The results obtained in the experiments with mitochondria were reproduced on inside-out SMP. In the SMP activation of the respiration by succinate leads to deprotonation of the certain probe fraction (Fig. 4A, spectrum 1). The uncoupler (FCCP) slightly diminishes this response (Fig. 4A, spectrum 2). The spectra observed in these experiments may result from the superposition of two effects: (i) the H^+ activity decrease in the outer-side membrane proteins and (ii) the H^+ activity increase in the inner-side membrane proteins. The latter effect dissipates more slowly than the former one; that enables to detach H^+ activity shift on the inner side after respiration restraint by TTFA (Fig. 4, spectrum 3). At high buffer concentrations in the incubation medium, both effects are reduced (Fig. 4B, spectra 1, 3). Therefore, buffer penetrates through the membrane of labelled SMP.

The results can be explained in terms of the local 'mosaic protonic coupling' model [8,9,18], which assumes that the membrane is anisotropic to H^+ transfer.

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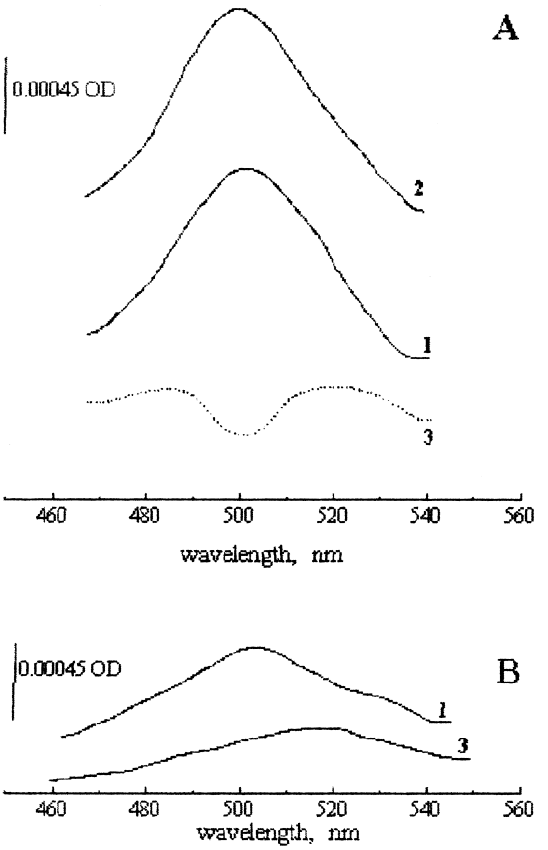

Fig. 4. Differential spectra of the FITC-labeled SMP after subsequent additions of (1) succinate; (2) FCCP (150 nM); (3) TTFA (5 μM). A: Incubation medium with low buffer concentration (a). B: Incubation medium with high buffer concentration (b).

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