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Role of Ca^{2+} in structure and function of Complex I from *Escherichia coli*

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ABSTRACT

The dependence of *E. coli* Complex I activity on cation chelators such as EDTA, EGTA, NTA and o-phenanthroline was studied in bacterial membranes, purified solubilized enzyme and Complex I reconstituted into liposomes. Purified Complex I was strongly inhibited by EDTA with an I_{50} of approximately 2.5 μM. The effect of Mg^{2+} and Ca^{2+} on EGTA inhibition of purified Complex I activity indicated that Ca^{2+} is tightly bound to the enzyme and essential for the activity. Low sensitivity to o-phenanthroline argues against the occupation of this cation binding site by Fe^{2+} or Zn^{2+}. The sensitivity of Complex I to EDTA/EGTA strongly depends on the presence of monovalent cations in the medium, and on whether the complex is native, membrane-bound, or purified. The data is discussed in terms of a possible loss either of an additional 14th, subunit of *E. coli* Complex I, analogous to Nqo15 in the *T. thermophilus* enzyme, or another component of the native membrane that affects the affinity and/or accessibility of the Ca^{2+} binding site.

1. Introduction

NADH:ubiquinone oxidoreductase type 1 (Complex I) constitutes the beginning of the respiratory chain in mitochondria and many bacteria. Even the "minimal" bacterial version consists of 6 hydrophilic and 7 hydrophobic subunits, and due to this complexity the knowledge of its structure and molecular mechanism is limited. Recently, several metal binding sites were revealed in the hydrophilic domain of *T. thermophilus* Complex I by analysis of its X-ray structure [1]. One of these sites is located between subunits Nqo15 and Nqo3, and is occupied by a tightly bound metal ion tentatively assigned as Ca^{2+}, which was suggested to stabilize the Nqo15–Nqo3 interaction. The other site(s) situated in an acidic groove of Nqo3 could also contain tightly bound Ca^{2+}. One further metal binding site formed by amino acid residues of the Nqo2 and Nqo1 subunits is located in the vicinity of the interface between Nqo15 and the rest of Complex I, which was considered as a putative iron-binding channel [2]; the metal bound there was identified as Mn^{2+}. However, it was suggested that this site may be occupied by Fe^{2+} in native conditions and that it may serve as an iron reservoir in case of damage to the neighbor FeS cluster, N1a [1]. The role of bound divalent cations in Complex I still remains unclear: the putative binding sites determined from the *T. thermophilus* Complex I structure are located between the other subunits and Nqo15, but so far this frataxin-like subunit was identified only in the *T. thermophilus* enzyme structure. The genes for homologous proteins were found only in the genomes of other thermophilic bacteria, which are close relatives of *T. thermophilus* [3], but not in *E. coli*. The question therefore arises whether these metal binding sites are constitutive for Complex I from different sources, or whether they are unique features of Complex I from thermophiles.

Recently, one zinc ion per molecule of mitochondrial Complex I was found by ICP emission spectrometry [4] and EXAFS [5]. The physiological role of Zn^{2+} in Complex I is unknown but it was suggested to be tightly bound because it was not released by EDTA treatment [4]. Since bound Zn^{2+} was not detected in the resolved structure of *T. thermophilus* Complex I it may be unique for the mitochondrial enzyme, or could be located in the membrane subunits that were not present in the crystal structure.

It is not known yet whether the bound divalent cations are intrinsic for the entire Complex I family and essential for structure/function. So far not much information about the interaction of these cations with Complex I is available, although they were shown to cause multiple effects on mitochondrial and bacterial Complex I. In the mitochondrial enzyme Ca^{2+} was found to attenuate GTP binding [6], and Mg^{2+} and Ca^{2+} prevented the Complex I transition from an inactive to the active state [7,8]. Bacterial Complex I is stabilized by Ca^{2+} [9], but its ubiquinone reductase activity is moderately inhibited by Mg^{2+} and Ca^{2+} in the millimolar concentration range [10].

We performed titrations of various *E. coli* Complex I activities in different states of the enzyme, and with a number of ion chelators. The results show that at least one tightly bound metal, most probably Ca^{2+}, is required to maintain the activity. The accessibility of this site to water-soluble chelators such as EDTA and EGTA, and/or the affinity to Ca^{2+}, depends on whether Complex I is solubilized or membrane-bound.

Abbreviations: DDM, n-dodecyl β-D-maltopyranoside; HAR, hexaammineruthenium (III) chloride; DQ, decylubiquinone; FeCy, ferricyanide; DEA, diethanolamine; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; NTA, nitrilotriacetic acid

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2. Materials and methods

2.1. Bacterial growth and purification of Complex I

The *E. coli* MWC215 (Sm^R *ndh*::Cm^R) strain was grown in an LB medium at 37 °C in a 25 L fermentor and harvested at the late exponential growth phase. The membranes for Complex I purification (large batch) were prepared by passing the cells through an APV Gaulin homogenizer as described in [11]. Complex I was purified in two chromatography steps, using anion exchanger DEAE-Trisacryl M (Bio-Sepra) columns and gel filtration on Superdex 200 prep grade (GE Healthcare) [10].

2.2. Measurements of catalytic activity

HAR and DQ reductase activities of purified or membrane-bound Complex I were measured by following NADH oxidation at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) [11] in the basic buffer containing 25 mM HEPES-BTP, pH 7.5, if not indicated otherwise. Concentrations of substrates added were 100 μM for DQ, 360 μM for HAR and 200 μM for NADH. For the measurements of the ubiquinone reductase activity of purified solubilized Complex I, the basic buffer was supplemented with 0.005% DDM and 20 nM ubiquinol oxidase bo_3. For the measurements of the activity of reconstituted Complex I, the basic buffer was supplemented with 10 μg/ml alamethicin and 20 nM ubiquinol oxidase bo_3. DQ reductase activity of purified Complex I was fully sensitive to rolliniastatin [12].

2.3. Analytical procedures

Protein concentrations in membranes were determined by the BCA protein assay reagent kit and for a purified protein Pierce 660 nm protein assay reagent was used (both from Thermo Scientific) Bovine serum albumin was used as a standard in both cases.

2.4. Membrane-bound Complex I preparations

A small batch of membranes was prepared from freshly grown cells by a fast protocol employing mild sonication of lysozyme treated, osmotically sensitive cells as described in [13]. Large batch membranes were obtained as described in [11] from the cells grown in fermentor, frozen and then disrupted by means of the APV Gaulin homogenizer. The small batch membranes are expected to be in a more native state than the membranes prepared from the cells grown in a fermentor where a fast preparation procedure and mild conditions are impossible.

2.5. Reconstitution of Complex I into liposomes and monitoring of ΔpH and Δψ generation upon NADH:DQ oxidoreduction

Asolectin at concentration 6 mg/ml was suspended in 200 mM MES-KOH buffer, pH 6.75, containing 0.5% DDM by sonication until clear, then purified Complex I was added to a concentration of 0.2–0.3 mg/ml. 1 mM of pyranine was added when the proteoliposomes were used for ΔpH monitoring. The mixture was allowed to equilibrate for 30 min at room temperature; then 400 mg/ml SM-2 BioBeads (Bio-Rad Laboratories), were added to remove the detergent. After 2 h at RT under gentle agitation the BioBeads were removed, the prepared proteoliposomes were diluted with 200 mM MES-KOH, pH 6.75, and pelleted by centrifugation at 170000 g for 90 min. The pellet was carefully washed and the proteoliposomes were suspended in a small volume of the same buffer. The orientation of reconstituted Complex I was tested by measurements of NADH: HAR oxidoreductase activity in the presence and absence of alamethicin. It was found that approximately 70% of Complex I was incorporated into the liposomes with the hydrophilic fragment outwards. The proteoliposomes were used for monitoring the Δψ by means of the electric potential-sensitive dye, Oxonol VI. Changes in absorption at 625–580 nm were measured with a Shimadzu dual-wavelength/double-beam UV3000 Spectrophotometer. The assay contained 200 mM MES-KOH, pH 6.75, and 0.5 mM $MgSO_4$, 100 nM monensin, 0.5 μM Oxonol VI, proteoliposomes (6–8 μg Complex I/ml) and 60 μM DQ. The reaction was started by 100 μM NADH addition. ΔpH generation was monitored by following the fluorescence changes of the water-soluble pH-indicator pyranine ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 460$ nm) entrapped in the proteoliposomes in the same buffer, but instead of monensin the medium was supplemented with 0.5 μM valinomycin.

3. Results

3.1. Non-linear DQ reductase activity

The curves of NADH consumption by DQ reduction were not linear for all tested Complex I preparations (Fig. 1A), which is much more evident when the derivatives of these curves, are depicted (Fig. 1B). The activity of the enzyme in the membrane preparations has no (small scale) or negligible (large scale) lag phase but after

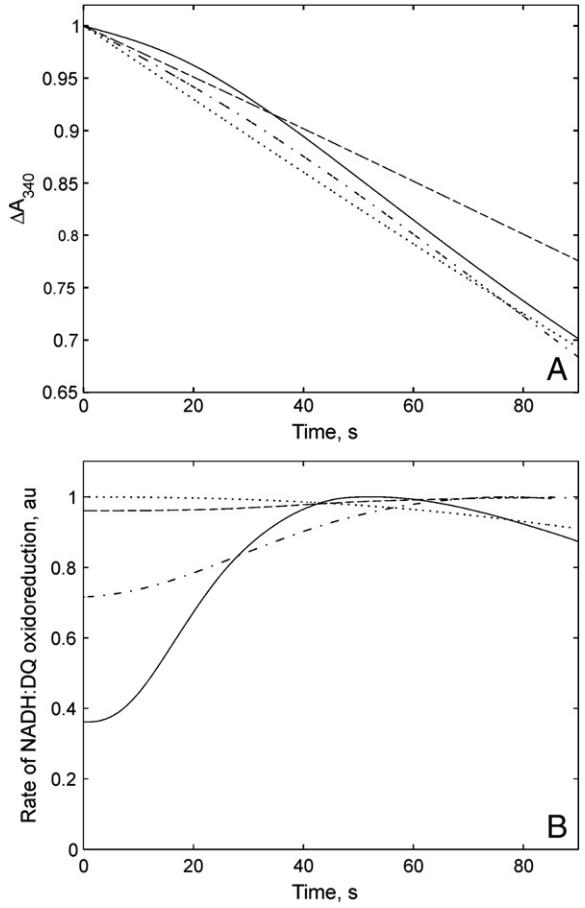

Fig. 1. Initial activity of Complex I in different states. A. NADH consumption upon NADH: ubiquinone oxidoreduction by Complex I in different preparations; NADH was added at zero time; B. derivatives of NADH consumption curves representing the rates of Complex I ubiquinone reductase activity. The curves were normalized by their maximal values: 1.6 and 1.7, large and small scale membrane preparations respectively, 26, purified solubilized Complex I, 35, purified reconstituted Complex I, μmol mg^{-1} min^{-1}. Membrane-bound Complex I, large scale membrane preparation, dotted line; small scale membrane preparation, dashed line; purified solubilized Complex I, solid line; purified reconstituted Complex I, dash–dot line.

approximately 45–80 s the rate started to decrease. The purified, solubilized Complex I was activated upon turnover, the rate of NADH oxidation was increased 2–3 times during 40 s, approached the maximal value and then decreased after 60 s (Fig. 1B). After reconstitution the lag phase was shorter, and the activation was 40–20% depending on the preparation However, the activation time was close to that for solubilized Complex I or longer; then the activity was also decreased. The observed lag phase is not very unusual: some membrane enzymes (e.g. succinate dehydrogenase [14] and cyt c oxidase [15]) are known to convert to an inactive state upon preparation and require turnovers for full activation. The decrease of the activity was most probably due to a slow exchange of the reduced ubiquinone between lipid membranes or detergent/protein micelles and the bulk solution and, therefore, ubiquinol accumulation in the enzyme milieu. To compare the activity of solubilized and reconstituted Complex I at different conditions the values of the activity in the interval 45–60 s (Fig. 1B) were averaged and plotted against the chelator concentration.

3.2. Effect of EDTA on the HAR and DQ reductase activity of membrane-bound, solubilized and reconstituted Complex I

We found that Complex I bound to native membranes is not very sensitive to EDTA. Artificial HAR reductase and natural DQ reductase activities were both decreased less than 10% by EDTA for small batch membranes (the membranes prepared for the best measurements of proton-pumping activity [13]), and by about 30% for large batch membranes obtained for Complex I purification from the cells grown in the fermentor (Fig. 2A). The drop of activity happened already upon addition of 5 μM EDTA; after that the activity stayed unchanged up to an EDTA concentration of 1–2 mM (not shown). The solubilized Complex I is much more sensitive to EDTA. Both activities drop by 75–80% (Fig. 2B). The half-effect was approached at the same concentration of EDTA of approximately 2.5 μM, as for the membranes. The effect of EDTA on reconstituted Complex I was intermediate, 50–60% activity was lost (Fig. 2C). However, when the liposomes were washed once they became as sensitive to EDTA as the solubilized enzyme.

The drop of the HAR and DQ reductase activities upon addition of chelators goes in parallel, but the artificial activity is 3–4 times higher than the natural one. This suggests that the EDTA treatment does not result in some gradual changes in the electron transfer but rather blocks almost completely the activity of a certain fraction of the enzyme. In line with this is the observation that even FeCy reductase activity of purified Complex I is depressed by EDTA, although to a lower extent, only 30–50% was lost (Supplementary Fig. 1S). Since there are strong indications that FeCy accepts electrons directly from FMN [16] the architecture of the input catalytic site of Complex I must be disturbed by the EDTA treatment. Although the reconstituted Complex I is highly sensitive to EDTA, almost as sensitive as the solubilized enzyme but differently from native membrane-bound Complex I, it is nevertheless competent in the translocation of H^+ across the membrane as verified by measurements of $\Delta\psi$ and ΔpH (Fig. 3).

3.3. Effect of NTA and o-phenanthroline on Complex I activity

To find out what metal is removed by EDTA, Complex I activity was titrated with other chelators. EDTA and EGTA, which are very similar in structure and binding properties, showed the same effect on Complex I activity although the affinity of EGTA for Ca^{2+} is higher. It could therefore be expected that EGTA should inhibit the enzyme at lower concentrations than EDTA. However, at such low concentration of the chelator (micromolar scale) kinetic factors become limiting. Within the time range of the reaction (Fig. 1) the divalent cation depletion of the enzyme by the chelators does not reach equilibrium.

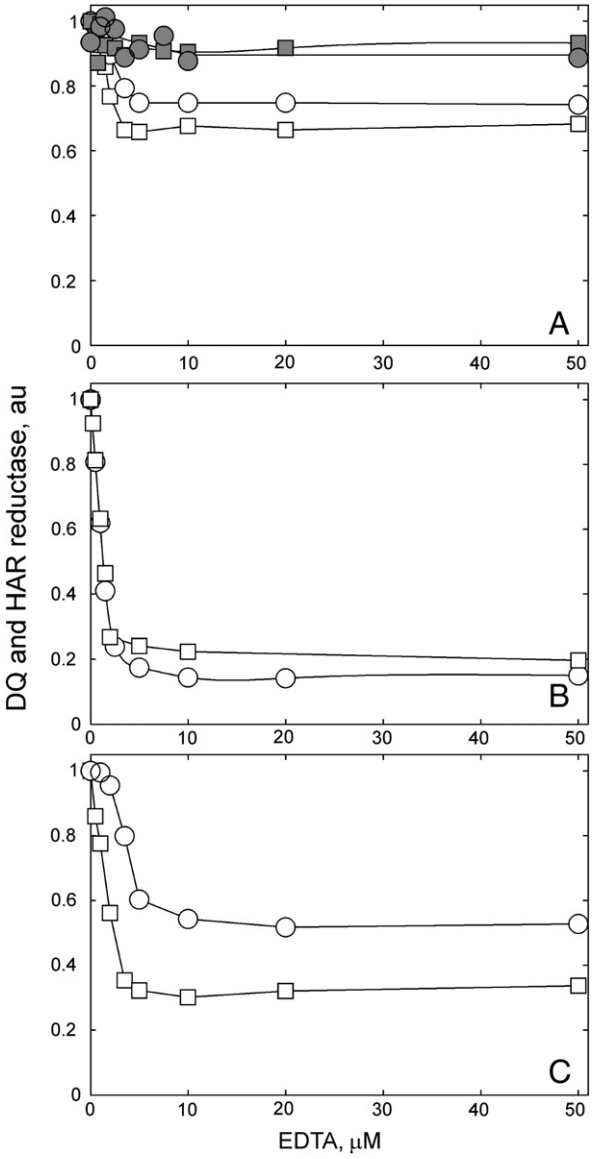

Fig. 2. Titration of Complex I NADH:HAR (squares) and NADH:DQ oxidoreductase (circles) activities by EDTA. A, Membrane-bound Complex I, small batch membranes specifically obtained for proton-pumping measurements, gray symbols, and large batch membranes obtained from cells grown in the fermentor, open symbols. B, Purified, solubilized Complex I. C, Purified Complex I reconstituted into the liposomes.

This is the limitation of the used approach which is why a proper determination of the I_{50} for EGTA was not possible. The preincubation with the chelators would release this time limitation, however the activity is measured at pH 7.5, but Complex I is highly unstable at pH higher than 6.0, which makes the preincubation unfeasible. NTA is efficient at a concentration more than 2 orders of magnitude higher (Fig. 4). Since NTA binds Mg^{2+} and Ni^{2+} with the same affinity as EGTA, but has much lower affinity for Zn^{2+} and Ca^{2+}, it appears that the metal bound to Complex I is one of the latter.

o-Phenanthroline is a strong chelator of Zn^{2+} and Fe^{2+} and is used for depleting the enzymes of Zn^{2+}. o-Phenanthroline was shown to be a weak inhibitor of only the quinone reductase activity of mitochondrial and *P. denitrificans* Complex I [17]. We observed the same effect on solubilized and membrane-bound Complex I from *E. coli*. o-Phenanthroline inhibited DQ reductase activity with a half-effect at 100 μM without any influence on HAR activity (not shown). Therefore, o-phenanthroline has most probably another target than

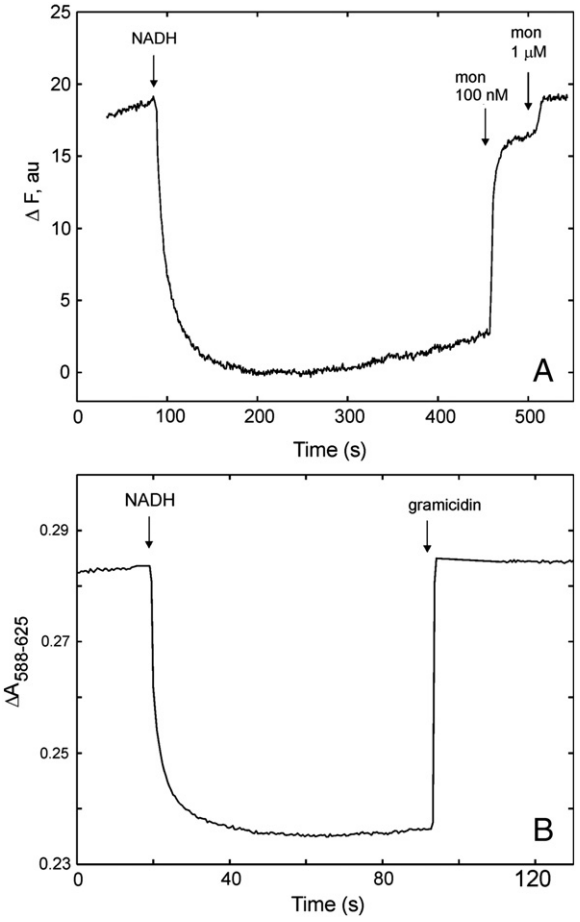

Fig. 3. Monitoring ΔpH and Δψ generation by Complex I reconstituted into liposomes. A. Generation of ΔpH, acidic inside, followed by fluorescence changes of pyranine entrapped in the proteoliposomes; ΔpH was dissipated by following additions of monensin 100 nM and 1 μM. B. Generation of Δψ, positive inside, was followed by means of Oxonol VI; Δψ was dissipated by the addition of 1 μg/ml gramicidin.

EDTA in Complex I; perhaps it binds to the quinone binding site as reported for a bacterial reaction center [18]. From this data we can conclude that the cation removed by EDTA is most likely Ca^{2+}.

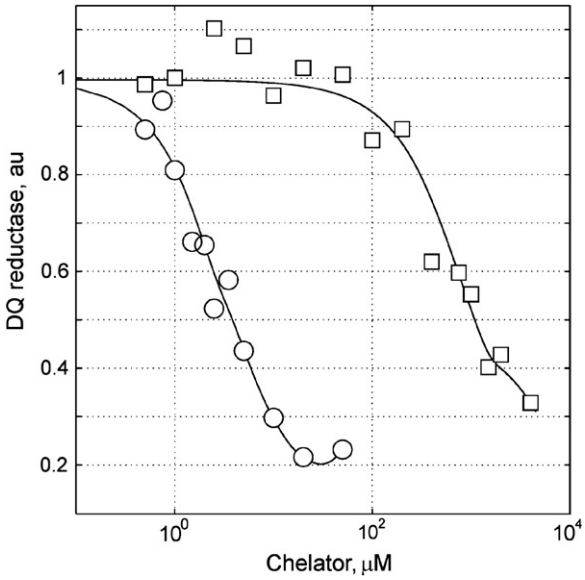

Fig. 4. Titration of NADH:DQ oxidoreductase activity of purified, solubilized Complex I with EGTA (circles) and NTA (squares).

3.4. Identification Ca^{2+} as a tightly bound divalent cation in Complex I

Although the effect of the chelators is irreversible, at least in the time scale of the experiments, it can be prevented. The prevention of inhibition of Complex I activity by EDTA or EGTA by addition of equivalent amounts of Mg^{2+} and Ca^{2+} (Fig. 5) also indicates that it is likely the Ca^{2+} that is tightly bound. The affinity of EGTA for Mg^{2+} is much lower than that of EDTA, which allows us to discriminate between bound Ca^{2+} and Mg^{2+} in the protein. In the presence of an equimolar amount of Mg^{2+} Complex I activity was only partially inhibited by 20 μM EDTA, and in the presence of an equimolar amount of Ca^{2+} the activity approached the control level without chelators. Upon inhibition by 20 μM EGTA the presence of 20 μM Mg^{2+} prevents the inhibition much less (Fig. 5), and the presence of 20 μM Ca^{2+} again returns the activity to the control level. The phenomenon was less prominent with quinone reductase activity (Fig. 5A), but clearly evident with HAR and FeCy reductase activities (Fig. 5B and Supplementary Fig. 2S), where there was practically no difference in EGTA inhibition whether Mg^{2+} was added to the test buffer or not. This data also makes it unlikely that a transition metal is responsible for this phenomenon. Transition metals have a much higher affinity for EDTA and EGTA; therefore Ca^{2+} should not completely restore the activity.

3.5. K^+ ions confer Complex I insensitivity to EDTA

It was found that the Complex I activity becomes chelator insensitive in the presence of K^+ (Fig. 6). Surprisingly, this effect

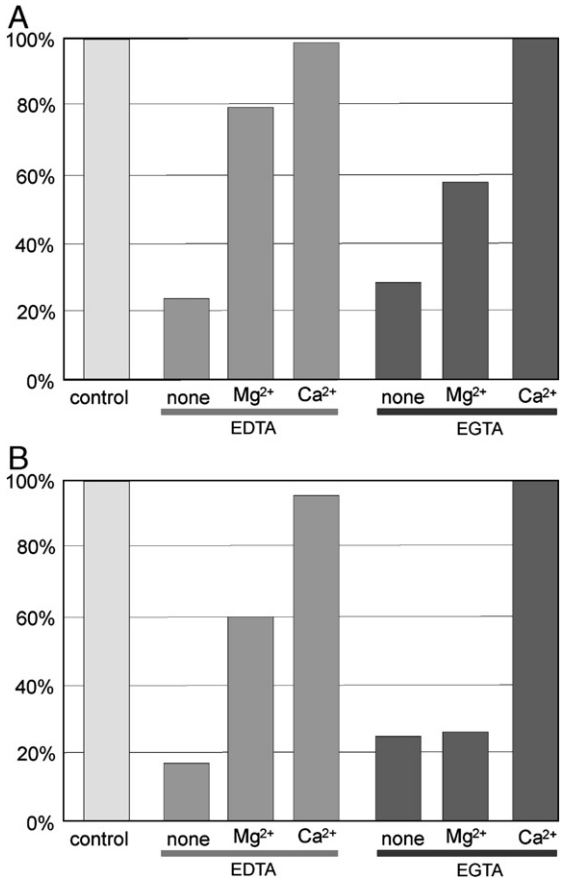

Fig. 5. Prevention of the chelators' inhibitory effect by Mg^{2+} and Ca^{2+}. The ubiquinone reductase (A) and HAR reductase (B) activities of solubilized Complex I inhibited by 20 μM EDTA or EGTA in the presence and absence of $MgCl_2$ and $CaCl_2$ at equimolar concentration as indicated. 100% activity corresponds to 110–150 for HAR reductase and 25–35 for DQ reductase μmol NADH mg^{-1} min^{-1}.

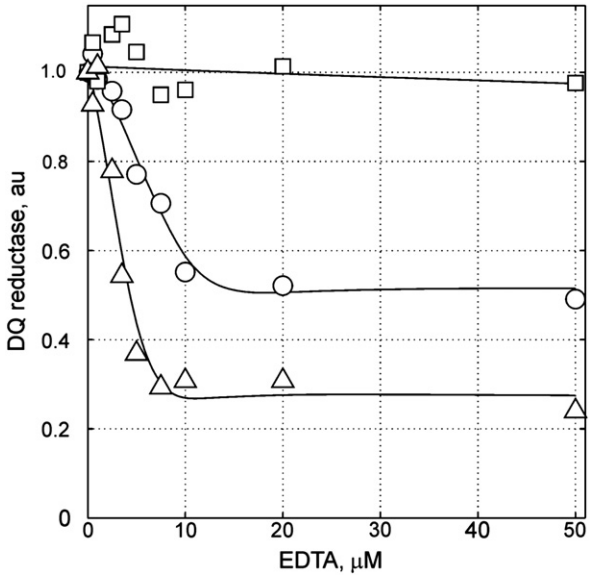

Fig. 6. Titration of DQ reductase activity of purified, solubilized Complex I in the presence of different monovalent cations by EDTA. The medium contained 100 mM of KOH (squares), NaOH (circles) or DEAOH (triangles) adjusted by HEPES to pH 7.5.

was not due to the ionic strength. Replacement of K^+ with Na^+ or Li^+ (not shown) resulted only in a partial effect whereas the organic cations DEA^+ and BTP^+ were practically inefficient. It is noteworthy that the stability of Complex I correlates with its sensitivity to EDTA; stability is much higher for a native membrane-bound enzyme than for solubilized Complex I, and K^+ ions specifically stabilize Complex I in all preparations (not shown).

4. Discussion

The reported data indicates that the inhibitory effect of the chelators on *E. coli* Complex I activity may best be explained by the removal of tightly bound Ca^{2+}. The possibility that the chelator anions may be inhibitory themselves cannot be completely excluded, but the action of NTA (Fig. 4), a chemically very different chelator, where inhibition is proportional to its affinity to Ca^{2+}, argues against this suggestion. The other finding that contradicts the effect of free EGTA is that inhibition of HAR reductase activity by 20 μM EGTA is practically unaltered regardless of the presence of 20 μM Mg^{2+} (Fig. 4B); after addition of the Mg^{2+} the concentration of free EGTA is negligible. These findings, together with the known Ca^{2+} binding site in the structure of the hydrophilic part of Complex I from *T. thermophilus* [1], make it very unlikely that the chelators would be inhibitory themselves.

Comparison to the cation binding sites found in the *T. thermophilus* Complex I structure is not straightforward. In the *T. thermophilus* Complex I structure tightly bound Ca^{2+} is located at the interface between two subunits, Nqo3 and Nqo15, which participate equally in the formation of the binding site. The former corresponds to NuoG in the *E. coli* enzyme, but the latter is absent. Another cation binding site in the *T. thermophilus* Complex I, which is located in a proposed "iron channel", and contains a cation identified as Mn^{2+}, is formed by amino acid residues from subunits Nqo1 and Nqo2 [1], but only one out of four metal ligands, S77 in NuoE (counterpart of S68 in Nqo2), is present in the *E. coli* enzyme. The other three ligands in the *T. thermophilus* enzyme do not have clear counterparts in *E. coli* and the region of their location based in the alignment is not conserved in the NuoE subunit. However, the cation binding site in NuoE may be formed by the side chains of different amino acid residues or even by the backbone, but in this case it would be difficult at first glance to explain how the site situated between subunits NuoF and NuoE, which are at a significant distance from the membrane, may be disturbed by Complex I solubilization and purification. A reasonable explanation of the observed phenomenon may be that Complex I from *E. coli* also has an additional 14th subunit, which is not itself important for catalysis, but which increases enzyme stability, as does Nqo15 in *T. thermophilus* [3]. If this additional subunit is bound more loosely than in the *T. thermophilus* enzyme, it may be washed out in the steps of enzyme purification yielding a low population in the purified enzyme. Loss of this subunit, which may shield the cation binding site, would result in increased accessibility of the site for the chelators and/or a decreased affinity for Ca^{2+}. In turn, the removal of Ca^{2+} may disturb the interaction between the NuoF and NuoE subunits and result in structural changes in the catalytic site. Nqo15 in *T. thermophilus* was characterized by its fold as a frataxin-like protein [2]. The gene for Nqo15 is not located in the *nqo* operon encoding 14 subunits of Complex I but in a locus separated from it by ~360 kb [3]. The operon *nuo* encoding the 13 subunits of Complex I in *E. coli* also does not contain an additional gene, and there is no gene homologous to the Nqo15 protein by primary amino acid sequence in the whole *E. coli* genome. However, there is a gene for at least one protein belonging to the frataxin family as judged by its fold, namely CyaY (PDB 1EW4 and 1SOY [19,20]). The exact function of CyaY is not clearly identified yet, but as a frataxin orthologue it is proposed to carry iron and pass it on to other proteins [20]. Earlier CyaY was suggested to be a component of the *E. coli* Complex I. However, this possibility was excluded by deletion of *cyaY* and by locating CyaY in the cytoplasm by means of live cell imaging [21]. The frataxin-like component of Complex I may be a unique property of thermophilic bacteria. On the other hand, it is probable that the sensitivity of purified Complex I to chelators results from a loss of a protecting Ca^{2+} binding site stability factor which is not a protein or a polypeptide, but another component of the native membrane. Specific lipids seem unlikely since the chelators affect not only the ubiquinone reductase activity but also the artificial HAR and FeCy reductase activities, which require the operation of only two subunits, NuoE and NuoF, located far away from the membrane. Further work is required to clarify the nature of the stability factor and it is in progress now.

Protection of Complex I from chelators by K^+ ions may be explained by the fact that K^+ is an essential component of bacterial and eukaryotic cell cytoplasm, always present at high concentrations. Therefore, we argue that all proteins operating in the cytoplasm are adapted to that condition. Some of these proteins, including Complex I whose soluble domain is in the bacterial cytoplasm, require a physiological K^+ concentration to maintain their correct conformation. Previously, we demonstrated a high-affinity K^+ binding site in Complex I that affected its activity [10]. However, it seems probable that the enzyme also contains multiple low-affinity K^+ binding sites preserving its conformation.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.09.002.

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