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Rapid purification of wildtype and mutant cytochrome *c* oxidase from *Rhodobacter sphaeroides* by Ni^{2+}-NTA affinity chromatography

David M. Mitchell, Robert B. Gennis*

School of Chemical Sciences, University of Illinois, Urbana, IL 61801, USA

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Abstract A rapid and highly efficient method of purifying the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* has been developed. This method relies upon a six-histidine affinity tag fused to the C-terminus of subunit I, which confers to the oxidase a high affinity for Ni^{2+}-nitrilotriacetic acid (NTA) agarose. The histidine-tagged oxidase can be purified rapidly and with high yield by one affinity chromatography step, starting with solubilized membranes. The purified oxidase is >95% pure and possesses structural and functional characteristics of the wildtype enzyme. The six-histidine tag can be easily added to pre-constructed site-directed mutants of subunit I, increasing the availability of purified cytochrome *c* oxidase mutants for biophysical and biochemical studies.

Key words: Cytochrome *c* oxidase; Ni-chelate chromatography; Membrane protein; Bioenergetics; *Rhodobacter sphaeroides*

1. Introduction

Cytochrome *c* oxidase is an integral membrane metalloprotein which catalyzes the reduction of oxygen to water and uses the free energy from this reaction to pump protons across the membrane. The molecular mechanism by which this enzyme couples oxygen reduction to proton translocation has been a subject of much study [1–3]. It is now recognized that the eukaryotic cytochrome *c* oxidase is a member of a large superfamily of heme-copper oxidases which includes numerous prokaryotic respiratory oxidases. Much of the recent progress in understanding the structure and function of cytochrome *c* oxidase is due to application of molecular genetics techniques to several of the bacterial heme-copper oxidases [3–5]. One such model system for studying the aa_3-type cytochrome *c* oxidase has been developed in *Rhodobacter sphaeroides* [3,6–11]. Site-directed mutagenesis studies of this oxidase have led to the identification of the amino acid ligands of the metal centers and have suggested possible roles of other residues in the proton pumping activity of the enzyme [3,11].

Recently developed kinetic methods, when combined with site-directed mutagenesis of key amino acid residues, are likely to provide useful insight into the catalytic mechanism of the oxidase [12–15]. An improved purification scheme has been developed for the aa_3-type cytochrome *c* oxidase from *R. sphaeroides*, with the aim to provide a convenient and reliable protocol to obtain a sufficient supply of both wildtype and mutant variants for such experiments.

A rapid and highly efficient affinity purification scheme is described, using Ni-nitrilotriacetic acid (NTA) agarose and a six-histidine tag fused to subunit I of the oxidase. The use of Ni-NTA agarose to purify histidine-tagged proteins has been widely exploited, though not usually for intrinsic membrane proteins (see, for example, [16,17]).

2. Materials and methods

2.1. Materials

All chemicals used were of reagent grade. Lauryl maltoside was obtained from Anatrace. Ni-NTA agarose was obtained from Qiagen. DNA oligonucleotides were synthesized by the University of Illinois Biotechnology Center (Urbana, IL). Vent (exo$^-$) polymerase for PCR was obtained from New England Biolabs.

2.2. Fusion of six-histidine sequence to C-terminus of subunit I

All DNA manipulations were performed using the plasmid pJS3 [18] with the *E. coli* strain NM522 [19]. The six-histidine sequence was fused to subunit I in two steps: (1) a unique restriction site (*Xho*I) was added to the end of the coding region of subunit I of cytochrome *c* oxidase from *R. sphaeroides*, directly after the last codon, using a two-step PCR mutagenesis technique [20] in which a single mutagenic oligonucleotide was used: 5′-GAA CGC GCG CCC GCC CAC **TCG AGC** TAG CCT TCT GAT GCC CTA-3′; (2) a double-stranded DNA cassette, containing six in-frame histidine codons followed by a stop codon, was ligated to this unique *Xho*I site. The oligonucleotide sequences were as follows: 5′-TCG AAC **CAT CAC CAT CAC CAT CAC** TGA CCC GGG ATG TA-3′; 5′-AGC TTA CAT CCC GGG TCA **GTG ATG GTG ATG GTG ATG** GT-3′. The resultant plasmid was called pJS3(X6H). This construct conserves the entire wildtype sequence of subunit I, simply adding the following amino acid sequence to the C-terminus: SNHHHHHH (Fig. 1).

2.3. Bacterial growth

The strain of *R. sphaeroides* which was used (JS100) contains a deletion of the *ctaD* gene coding for subunit I of the aa_3-type cytochrome *c* oxidase [6]. The strain carries a derivative of the plasmid pRK-415 [21] containing the modified *ctaD* gene encoding the histidine-tagged subunit I. The cells were grown and harvested as previously described [7,18].

2.4. Preparation of solubilized membranes

Harvested cells are resuspended in an equal volume of 50 mM Tris, pH 8.0, (plus 50 μg/ml DNase I), and broken by passing through a French press at 20000 psi. Cell debris is removed by centrifugation at 12000 × *g* for 15 min. The supernatant is removed, and the membranes are pelleted by centrifugation at 185000 × *g* for 90 min. The membrane pellet is rehomogenized in about 5 volumes of 10 mM Tris, 40 mM KCl, pH 8.0. Lauryl maltoside is added to a final concentration of 1% for solubilization, and the membranes are stirred at 4°C for 15 min. The solubilized membranes are then centrifuged at 75000 × *g* for 30 min, and the supernatant is removed. The total amount of oxidase present in the membranes is estimated from the dithionite-reduced minus ferricyanide-oxidized spectrum ($\Delta A_{606-630}$ = 24 mM^{-1}) [7].

*Corresponding author. Fax: (1) 217-244-3186.
E-mail: gennis@aries.scs.uiuc.edu

Abbreviations: NTA, nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EPR, electron paramagnetic resonance; UV/VIS, ultraviolet/visible; PCR, polymerase chain reaction; ICP-AES, inductively-coupled plasma-atomic emission spectroscopy.

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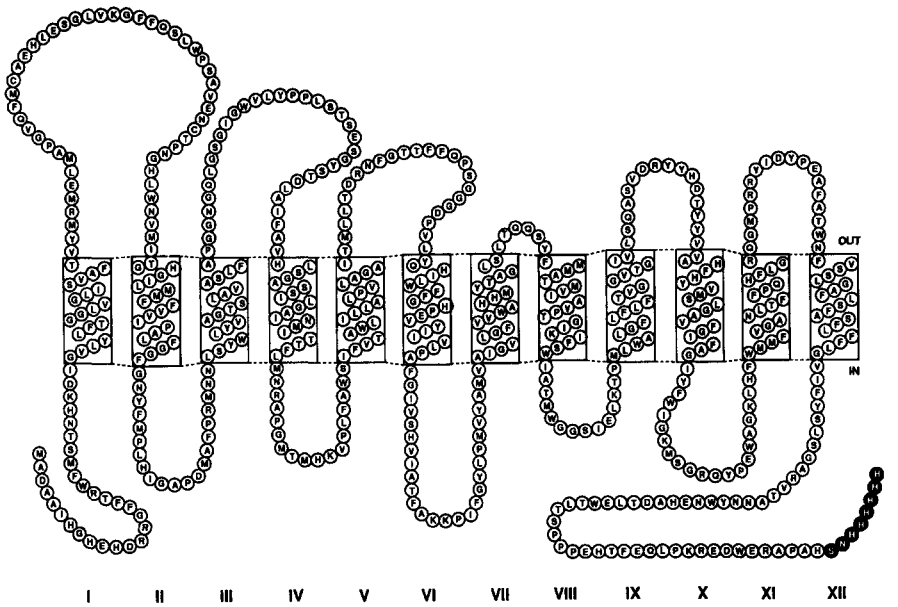

Fig. 1. Topological model of histidine-tagged subunit I of *R. sphaeroides* cytochrome *c* oxidase, showing 12 predicted membrane-spanning regions. The highlighted residues at the C-terminus were added in order to form a six-histidine affinity tag.

2.5. Ni^{2+}-chelate chromatography

Imidazole is added to the solubilized membrane solution to a final concentration of 10 mM. The Ni-NTA resin is then added (0.5 ml/mg oxidase), and the mixture is stirred at 4°C for 1 h. The mixture is then loaded onto a gravity-flow column, and washed with 10 column volumes of 10 mM Tris, 40 mM KCl, 10 mM imidazole, 0.1% lauryl maltoside (5 ml/min). This is followed by 5 column volumes of 10 mM Tris, 40 mM KCl, 20 mM imidazole, 0.1% lauryl maltoside (5 ml/min). The oxidase is eluted using a buffer containing 10 mM Tris, 40 mM KCl, 100 mM imidazole, 0.1% lauryl maltoside at 0.1 ml/min.

3. Results and discussion

The histidine-tagged enzyme is eluted from the Ni-NTA agarose with 100 mM imidazole as a three-subunit complex, as shown by SDS-PAGE in Fig. 2. Addition of 10 mM imidazole to the solubilized membrane solution during the binding to the Ni-NTA resin eliminates a large proportion of the non-specific and low-affinity binding of contaminants to the resin, increasing the binding capacity of the resin to approximately 2 mg oxidase per milliliter of Ni-NTA. The efficiency and selectivity of the Ni-NTA resin for the histidine-tagged oxidase is demonstrated by comparing the visible spectra and heme content of the membrane extract before and after binding to the resin (Fig. 3). Greater than 90% of the cytochrome *c* oxidase present in the solubilized membranes is recovered upon elution, with almost 100-fold increase in specific activity. Heme analysis using the pyridine hemochrome method [22] shows no contamination by cytochromes *b* or *c*. The entire purification scheme, from harvested cells to purified protein, can be performed in less than 8 h, and yields approximately 0.6 mg of oxidase per liter of cell culture.

Due to the high selectivity of the histidine tag, contaminants are efficiently removed. Apparently, there are no other proteins in the membranes of *R. sphaeroides*, expressed under aerobic growth conditions, which have a strong affinity for the Ni-NTA resin. It has been suggested that the cytochrome *c* oxidase of *Paracoccus denitrificans*, which is very similar to that of *R. sphaeroides*, exists as a supercomplex of proteins, including the bc1 complex and a membrane-bound cytochrome *c* [23]. If this were the case for the aa_3-type oxidase from *R. sphaeroides*, it might be expected that the histidine-tagged cytochrome *c* oxidase might co-purify with these other components. However, this is not observed under the conditions used in this purification, despite the relatively low detergent concentrations and mild treatment used. It is, therefore, unlikely that these enzymes exist tightly bound as a supercomplex in *R. sphaeroides*.

Photosynthetic pigments expressed by *R. sphaeroides*, which tend to interfere with ion exchange column purification of cytochrome *c* oxidase [7], have no apparent effect on the binding or purity of the histidine-tagged oxidase, even when these pigments are expressed at very high levels. This is especially significant for the purification of inactive oxidase mutants, where it is often observed that the membranes contain substantially higher levels of such pigments in comparison to the membranes containing the wildtype enzyme.

The Ni^{2+}-chelate method, by virtue of being both rapid and gentle, facilitates the purification of mutants that are unstable

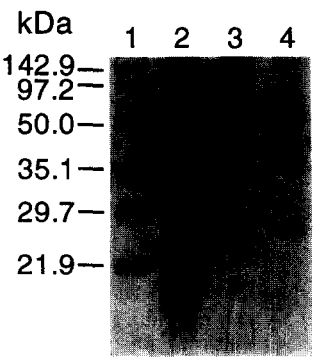

Fig. 2. SDS-polyacrylamide gel analysis at different stages in the purification. Lane 1 = molecular weight markers; lane 2 = membrane extract; lane 3 = 20 mM imidazole wash; lane 4 = elution with 100 mM imidazole.

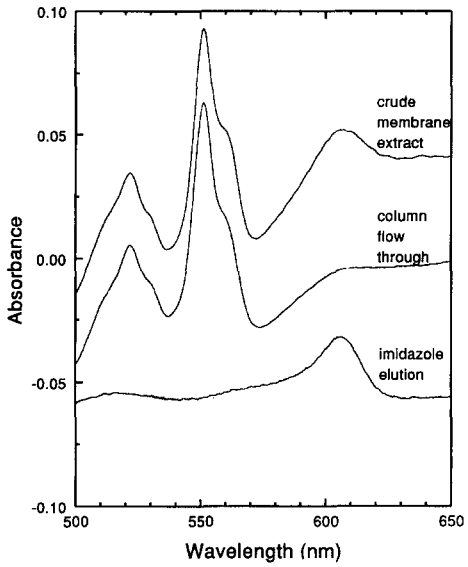

Fig. 3. Dithionite-reduced-minus-air oxidized difference spectra of (A) solubilized membranes extract; (B) column flow-through; (C) fraction eluted by 100 mM imidazole.

to purification by conventional methods. For example, the unstable mutants in which the histidine ligands of the low spin heme a have been altered (H102N and H421N) [18] were purified rapidly after having been fused to the six-histidine affinity tag, despite their low levels of expression (not shown).

In the current construct, the six-histidine tag has not been removed from subunit I. There is no evidence that this modification affects the structure or function of the enzyme. The oxidase turnover rate is in agreement with previously reported values (V_{max} = 1550 s^{-1}) [7]. Measurements of proton translocation performed in phospholipid vesicles demonstrate wildtype H^+/e^- ratios (John Fetter, unpublished results). Spectroscopic characterization, including UV/VIS, resonance Raman (Michelle Pressler and Younkyou Kim, unpublished results) and EPR spectroscopies (Roland Aasa, unpublished results), demonstrate wildtype structural features. Low-temperature Fourier-transform infrared absorbance difference spectroscopy of the enzyme-CO adduct also demonstrates wildtype characteristics (not shown). In addition, measurements of the internal reverse electron transfer rates [15] have demonstrated wildtype rates and extents of electron transfer upon photolysis of the CO-bound mixed valence enzyme (Pia Ädelroth, unpublished results). It has been noticed that in the presence of high concentrations of imidazole used to elute the oxidase from the Ni-NTA column, the α-band of the reduced-minus-oxidized spectrum is slightly blue shifted. This shift is reversed upon removal of the imidazole by dialysis or gel filtration chromatography, which is routinely done following the Ni-NTA affinity chromatography.

The purified enzyme was analyzed for metal content using ICP-AES. The Fe content is consistent with the heme A quantitation measured by the pyridine hemochrome method [22]. The Cu/Fe ratio was found to be 1.2, which is slightly lower than the value expected for a 3:2 Cu:Fe stoichiometry for the enzyme. Nevertheless, it is likely that the *R. sphaeroides* oxidase possesses a binuclear CuA center, as proposed for other bacterial and mitochondrial oxidases [24–26].

In summary, the data clearly demonstrate that histidine tagging can be a very effective technique for purifying intrinsic membrane proteins. The one-step protocol for purifying the *R. sphaeroides* aa_3-type cytochrome *c* oxidase will be a significant aid in furthering the biochemical and biophysical characterization of this enzyme.

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