Introduction

In the present work, we discuss the discovery and study of a novel cytochrome *c* from the purple sulfur bacterium *Thiocapsa roseopersicina*. We have characterized the cytochrome through a combination of absorption spectroscopy and circular dichroism (CD) with redox potentiometry and also by using differential scanning calorimetry (DSC). The data gathered place this protein in the c_4 class of cytochromes. We have used Mass Spectrometry to determine its primary structure, which confirmed this cytochrome to be of the c_4 type. It is the first purified and identified cytochrome c_4 from an anaerobic phototrophic bacterium.

Aims of the study

To purify and characterize proteins with redox centers from purple sulfur photosynthetic bacteria in order to shed some light over the electron transport pathways within the metabolism of these organisms.

Particularly, to characterize a novel periplasmic soluble cytochrome *c* from *Thiocapsa roseopersicina*:

- To determine its primary structure, by using the recent advances in Mass Spectrometry.
- To determine the heme content of the cytochrome, and the respective midpoint potential of each redox site.
- To determine its sensitiveness to oxygen, given that the organism is photosynthetically active only under anaerobic conditions, but can survive in aerobic conditions; and to determine its thermal stability, given that temperature is another sensitive parameter to the survival of the organism. In sum, to elucidate the interrelation of these two parameters in their influence on the cytochrome structure.
- To elucidate the phylogenetics of this protein.

Finally, to use all of the gathered structural information in order to clarify the function of this cytochrome and on a broader sense to gain insight into the electron transport chains of phototrophic bacteria.

Materials and Methods

The protein components from the cells of *Thiocapsa roseopersicina* strain BBS were extracted in a procedure that employed cold acetone. A first round of anion exchange batch liquid chromatography was followed by four rounds of alternating hydrophobic and anion exchange fast protein liquid chromatographies (FPLC). The purity and molecular weight were determined by SDS–PAGE.

The pyridine hemochromogen method was used, in combination with the Bradford method, to determine the heme content and the exact protein concentration.

In preparation for mass spectrometry, four different digestion sets were obtained by digestion of the protein with three different proteolytic enzymes. All measurements were performed on a linear IT-FTICR hybrid instrument. In most measurements, the proteolytic fragments of each digestion set were separated by online gradient reversed-phase micro-Liquid Chromatography prior to NSI, full-scan mass spectra were recorded in the FTICR unit, and MS/MS product ion scans in the IT. Most peptide sequences were determined by interpretation of the MS/MS spectra with the aid of a computer program.

The redox potential of the cytochrome was determined by potentiometric titration using a home-made stirred spectroelectrochemical cell which included a three electrode system. The enforced solution potential between the working and the platinum electrodes was provided by a potentiostat.

CD spectra were measured both in the far-UV range (190–250 nm) and near-UV–visible range (250–700 nm) as a function of the temperature in both anaerobic and aerobic conditions. CD spectra in the range 190–250 nm were used for calculation of secondary structures.

UV-VIS absorbance measurements and DSC measurements were performed to provide additional information in temperature dependence studies.

Results and Discussion

Cytochrome c_4 contains two hemes per molecule, as determined by the pyridine hemochromogen method, and later confirmed by Mass Spectrometry.

After analysis by MS of the peptide mixtures resulting from the proteolytic digestions, we have compiled the most abundant ions into comprehensive lists of peptide masses. Each peptide was then individually sequenced by interpretation of the respective MS/MS data. It was fairly straightforward to determine the sequence of peptides up to 14 amino acids in length (which translates to roughly 1500 Da in peptide mass). Larger peptides posed mounting difficulties. Linking up yielded the complete amino acid sequence of cytochrome c_4 from *Thiocapsa roseopersicina*. To our knowledge, this is currently the largest protein that has been completely sequenced by mass spectrometry alone.

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TDGHQAAAPQ VGDPQAGEAK ANGVCLACHG PQGNSLVPLW PKLAGQHPEY
IVKQLMDFKQ RRANEQMTPM AMPLTDQEVL DLAAYYATQP KTPGAADPEL
ASKGESLYRW GNPETGVPAC SGCHGPAGGA GQSLAKFPRL SAQHADYTKQ
TLEHFRGALR ANDPNGMMRG AAARLSDQEL AAVSQYLQGL SQ
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The direct mass spectrometry strategy used in this work can be often faster and more straightforward than the indirect gene sequencing approach.

Cytochrome c_4 proved to be a heat-tolerant protein if maintained under anaerobic conditions. We clarified that oxygen initiates an irreversible unfolding of the protein at high temperatures, most likely through direct binding to the heme's sixth coordination site, which was left vacant after dissociation of the native methionine ligand. It was shown that the methionine-Fe bond (i. e. the 6^{th} axial ligation) is closely linked to the protein moiety (i.e. the protein's secondary structure) and plays a crucial role in the overall folding of the protein.

Cytochrome c_4 proteins are thought to participate in aerobic respiratory pathways, in a position close to the terminal oxidase of the electron transport chain. The discovery of such a cytochrome in an anaerobic photosynthetic organism throws doubt upon this assumption. In *T. roseopersicina*, cytochrome c_4 must participate in photosynthesis instead. More specifically, since the redox titration revealed midpoint potentials of 237 ± 5 mV (1^{st} heme) and 268 ± 6 mV (2^{nd} heme), we suggest to place this cytochrome in the position of electron transport between cytochrome b/c_1 and the tetraheme cytochrome of the reaction centre.

Our phylogenetic survey revealed that cytochrome c_4 is widespread across γ-proteobacteria, and thus it is likely to have this role of periplasmic electron carrier in several species of photosynthetic bacteria.

In purple non-sulfur bacteria, monoheme cytochrome c_2 usually carries out this function, hence it is appealing to speculate that purple sulphur bacteria use instead a diheme cytochrome c_4 in order to link sulphur metabolism with the photophosphorylation cycle.

Summary of novel findings

- We have purified a novel cytochrome from the organism *Thiocapsa roseopersicina*. This was the first purified and identified cytochrome c_4 from a photosynthetic bacterium.
- We have determined the primary structure of cytochrome c_4 from *T. roseopersicina*. This was the largest protein (about 21 kDa in size) whose amino acid sequence has been determined solely by Mass Spectrometry.
- Using recent developments in the field, we have implemented a protein sequencing methodology that allows high-resolution Mass Spectrometry to compete with indirect DNA-based methods.
- We have characterized cytochrome c_4 from *T. roseopersicina* in terms of UV-Vis and CD spectroscopy, correlating spectral profiles to structural features of the protein.
- We have determined the midpoint redox potential of the two heme groups of cytochrome c_4 from *T. roseopersicina*.
- We have discovered that cytochrome c_4 is heat tolerant and indeed shows a very resilient secondary structure (especially the reduced state) if under anaerobic conditions, which are the normal growth conditions for *T. roseopersicina*. If under aerobic conditions and in oxidized state, the secondary structure and thus the overall fold of the protein is irreversibly affected by heating in a process where oxygen binding to the vacant 6^{th} axial positions of the heme groups plays a decisive role.
- Our survey through the phylogenetics of cytochrome c_4 from *T. roseopersicina* has revealed that this type of cytochrome is widespread in γ-proteobacterial organisms, particularly in Oceanospirillales and Chromatiales orders.
- Up until now cytochromes c_4 were believed to participate in oxygenic electron transport chains. In contrast, the work here presented indicates that in purple sulphur bacteria (Chromatiales), cytochrome c_4 plays a role in anaerobic phototrophic electron transport chains instead.

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