Dynamics in electron transfer protein complexes

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Chapter 2

Interaction of L-galactono-γ-lactone dehydrogenase with cytochrome *c*

Abstract

L-Galactono-γ-lactone dehydrogenase (GALDH) catalyzes the terminal step of vitamin C biosynthesis in plant mitochondria, the oxidation of L-galactono-1,4-lactone into L-ascorbate with the concomitant reduction of cytochrome *c* (Cc). Electron transfer between GALDH and Cc presumably involves the formation of a complex between the two redox partners. A surface engineering strategy was followed to identify the role of charged residues on the surface of *Arabidopsis thaliana* GALDH. In total three patches of Lys and Glu residues in close proximity were identified and mutated to Ala. Using heteronuclear nuclear magnetic resonance (NMR) spectroscopy transient complex formation was demonstrated between GALDH and a 9-fold surface mutant with isotopically enriched ferric yeast Cc. Chemical shift perturbations of ^{1}H and ^{15}N nuclei of ferric yeast Cc, arising from the interactions with unlabelled GALDH, were used to map the interacting surface of yeast Cc. The residues involved in interaction with GALDH are confined to a single surface patch around the haem edge of Cc. The dissociation constants for wild-type GALDH and its 9-fold surface mutant have been determined to be 50 ± 10 μM and 44 ± 10 μM respectively. The interaction with Cc remains intact after removing 9 charged residues at the GALDH protein surface. On the basis of the small size of the chemical shift perturbations, it is concluded that the complex is transient and of dynamic nature.

Introduction

The mitochondrial flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH; L-galactono-1,4-lactone: ferricytochrome *c* oxidoreductase, EC 1.3.2.3) completes vitamin C (L-ascorbate) biosynthesis in plants. GALDH is a so-called aldonolactone oxidoreductase and belongs to the vanillyl-alcohol oxidase (VAO) flavoprotein family[64,65]. Members of this family share a conserved FAD-binding domain and a less conserved cap-domain. The latter determines the substrate specificity. Most VAO-members are hydrogen peroxide producing oxidases, plant GALDH, however, exclusively uses cytochrome *c* (Cc) as electron acceptor[66]. GALDH is localized in the mitochondrial intermembrane space where it is involved, besides from producing L-ascorbate, in shuttling electrons into the respiratory chain via Cc and respiratory complex I[104-106]. The highly soluble Cc is a strongly conserved haem-containing component of the electron transport chain, in which it transfers electrons between the membrane bound Cc reductase and Cc oxidase complexes[107].

Electron transfer between GALDH and Cc presumably involves a physical interaction between both redox partners. Due to the charged nature of Cc (p*I* ~9-11) electrostatic interactions are thought to play an important role in complex formation. Cc contains a patch of positively charged residues surrounding the haem edge, the part of the surface where the haem cofactor is most accessible[108]. No crystal structure is available for GALDH or its homologues, so no information is available about possible interaction sites on the protein surface. The GALDH sequence, however, contains an above average number of charged residues, mostly Glu and Lys, which could be involved in surface interactions. Large, surface exposed hydrophilic residues often occur in patches of 2-3 residues in close proximity[109] and their replacement by Ala has been shown to improve protein crystal quality[110].

Previously, Leferink and co-workers (personal communication) were unable to detect any interaction between *Arabidopsis thaliana* GALDH and bovine heart Cc by size-exclusion chromatography[66]. This raised the possibility of transient interactions[111], which are likely influenced by the surface properties of both proteins. To gain insight into this matter, NMR chemical shift perturbation analyses were performed on wild-type

GALDH and a GALDH 9-fold surface mutant using ^{15}N-labelled ferric yeast Cc. The residues on yeast Cc involved in the interaction with GALDH and the 9-fold surface mutant have been highlighted and the dissociation constants of the complexes have been determined.

Materials and Methods

Protein preparation

The unlabelled wt-GALDH and its 9-fold surface mutant were provided by Dr. Nicole G. H. Leferink and Prof. Dr. Willem J. H. van Berkel from Wageningen University, The Netherlands[66]. The isotopically-enriched ^{15}N Cc was produced in *E. coli* and purified as reported previously[112,113].

Preparation of NMR samples

GALDH with a UV-vis peak ratio A_{276} / A_{450} of 8.15 and ferric Cc with a UV-vis peak ratio A_{410} / A_{280} > 4.0 were used for the NMR experiments. The protein concentrations of the wild type GALDH and its 9-fold mutant, and Cc were determined according to the absorbance peak at 450 nm (ε = 13.25 $mM^{-1}cm^{-1}$) and 410 (ε = 106.1 $mM^{-1}cm^{-1}$)[114] respectively. The NMR samples (0.5 ml) contained 0.14 mM unlabelled in 20 mM sodium phosphate pH 7.4, 6% D_2O for lock, and 0.1 mM $CH_3CO^{15}NH_2$ as internal reference. Microlitre aliquots of the stock solution of the ^{15}N-labelled ferric Cc (1.57 mM) were added. The titration consisted of 13 experimental points with 0, 20, 35, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 300 nmoles of the ^{15}N-labelled Cc. The pH of the samples was checked before and after each titration step and adjusted, if necessary, with small aliquots of 0.1 M NaOH or 0.1 M HCl solutions.

NMR experiments

The NMR experiments were performed at 303 K on a Bruker DMX600 spectrometer equipped with TCI-Z-GRAD cryoprobe (Bruker, Karlsruhe, Germany). For each titration, 1D and 2D [^{15}N, ^{1}H] HSQC spectra were acquired. The spectral widths (in Hz)

for 2D [^{15}N, ^{1}H] HSQC spectra were 1520.45 and 8090.61. All 2D spectra were acquired with 1024 and 80 complex points in the ^{1}H and ^{15}N dimensions, respectively. Data processing of the 1D ^{1}H and 2D [^{15}N, ^{1}H] HSQC spectra were performed in XWINNMR (Bruker) and AZARA 2.7 (http://www.bio.cam.ac.uk/azara/), respectively. The amides not observed in the present work were A3, E21, H26, V28, N31, H33, G34, H39, G45, S47, N56, G77, M80, G83, G84, K86, K87 and E88 for ferric Cc. Chemical shift perturbations of ^{15}N and ^{1}H nuclei were analysed by overlaying the spectra in Ansig-for-Windows[115].

Chemical shift titration curves were analysed with a two-parameter non-linear least square fit using a one-site binding model:

$$\Delta\delta = \tfrac{1}{2}\Delta\delta_0\left[A - \sqrt{A^2 - \frac{4}{R}}\right] \qquad (2.1a)$$

$$A = 1 + \tfrac{1}{R} + \frac{[Cc]_0 + R[GALDH]_0}{R[Cc]_0[GALDH]_0 K_B} \qquad (2.1b)$$

where $[GALDH]_0$ is the starting concentration of GALDH in the tube, $[Cc]_0$ is the stock concentration of Cc, R and $\Delta\delta$ are the ratio of the total concentrations of Cc and GALDH, and the chemical shift change at a given step in the titration, respectively, $\Delta\delta_0$ is the change in the chemical shift for 100% bound Cc and K_B is the binding constant. In the fits $\Delta\delta$ and R were the dependent and independent variables, respectively, and $\Delta\delta_0$ and K_B the fitted parameters.

Results

A general strategy to improve crystal quality involves the removal of large surface exposed hydrophilic residues[110]. The 9-fold mutant was designed to improve the crystal quality of GALDH which readily forms crystals diffracting to about 3.5 Å resolution (N. G. H. Leferink, E. Carpanelli, A. Mattevi & W. J. H. van Berkel, personal communication). The GALDH sequence contains an above average number of Lys and Glu residues (9.1% and 7.2% respectively, vs. 5.8% and 6.1% respectively, on

average,[116]), distributed over the entire sequence. Surface exposed patches of Lys and Glu residues were assigned based on their predicted position in a 3D homology model of GALDH (Figure 2.1). Three patches of Glu and/or Lys residues were mutated into Ala resulting in one 9-fold surface mutant (E44A-K45A-K46A, FAD domain; E83A-K84A-K85A, FAD domain; K245A-K247A-K249A, cap domain).

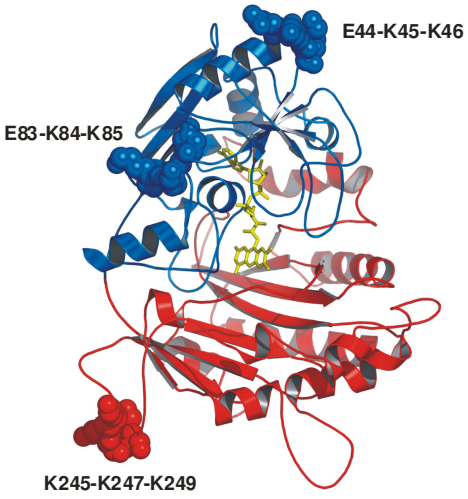

Figure 2.1. Ribbon diagram of a 3D model of GALDH with the predicted patches of surface exposed residues. The predicted FAD-binding domain is in blue and the cap-domain in red. The mutated patches of surface residues are represented as spheres, the FAD-cofactor is shown in yellow sticks. The GALDH homology model was prepared with cholesterol oxidase (PDB entry 1I19;[117]) and alditol oxidase (PDB entry 2VFS;[118]) as template structures using the programme Modeller[119,120]. The model was made by Dr. Nicole G. F. Leferink and Prof. Dr. Willem J. H. van Berkel (Wageningen University, Netherlands).

The interaction of GALDH and the 9-fold surface mutant with ferric yeast Cc was studied in reverse titration experiments, varying the concentration of ^{15}N-labelled Cc. Complex formation was evidenced by an increase in line-width for all peaks in the NMR spectrum, as well as chemical shift perturbations of certain resonances. For all titrations the magnitude of the line broadening in the bound form was the same for both shifted and unaffected peaks, and a single set of amide peaks in the 2D [^{15}N, ^{1}H] HSQC spectrum was observed. This indicates that the GALDH – Cc complex is in fast

exchange on the NMR time scale (k_{off} > 125 s^{-1}). Chemical shift perturbations for the amide resonances were monitored in a series of 2D [^{15}N, ^{1}H] HSQC spectra.

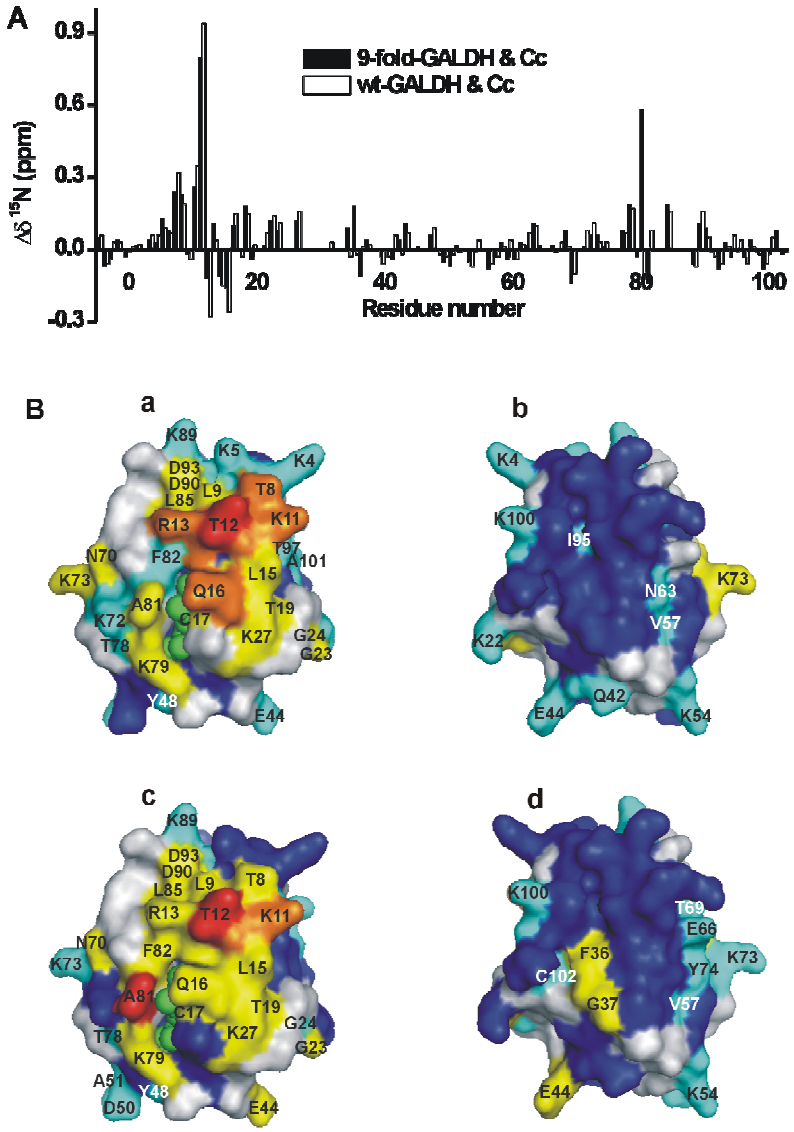

Figure 2.2. **(A)** Cc amide chemical shift perturbations ($\Delta\delta^N$) for the Cc – GALDH complexes. **(B)** Chemical shift perturbations ($\Delta\delta^N$) of ferric Cc upon binding with GALDH, mapped on a surface representation of Cc (PDB entry 1YCC): $\Delta\delta^N$ > 0.5 ppm in red, $\Delta\delta^N$ > 0.25 ppm in orange, $\Delta\delta^N$ > 0.10 ppm in yellow, $\Delta\delta^N$ > 0.05 ppm in cyan and $\Delta\delta^N$ < 0.05 ppm in blue. Unassigned and proline residues are shown in grey, and the haem is

coloured green. **a** and **b** represent the front side and back side of Cc in complex with wt-GALDH, **c** and **d** represent the front side and back side of Cc in complex with 9-fold surface mutant of GALDH.

The chemical shift perturbations in the ^{15}N dimension ($\Delta\delta^N$) were more significant than those in the ^{1}H dimension ($\Delta\delta^H$). The $\Delta\delta^N$ values for Cc (0.15 mM) in the presence of 0.14 mM wild-type GALDH and the GALDH 9-fold surface mutant are presented in Figure 2.2A. Based on the size of the chemical shift perturbations, the Cc residues involved in interaction with GALDH and its 9-fold mutant have been identified and the interaction surface of Cc has been mapped. The Cc residues interacting with GALDH and its 9-fold surface mutant are confined to the haem edge of Cc, regarded as the front side in this work (Figure 2.2B). Thr12 showed the largest chemical shift perturbation. The chemical shift perturbations for this residue were plotted against the Cc/GALDH ratio and the titration curves were fitted to a 1:1 binding model (Figure 2.3). From these titration curves, the values of K_d and $\Delta\delta_0$ were calculated, yielding 50 ± 10 µM and 0.75 ± 0.04 ppm, respectively, for wild-type GALDH and 44 ± 10 µM and 0.93 ± 0.05 ppm for the 9-fold surface mutant.

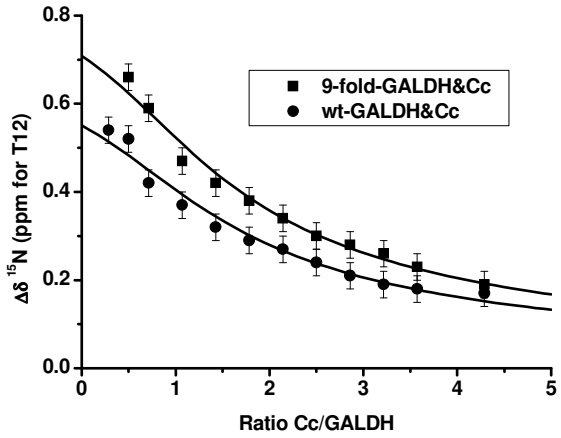

Figure 2.3. Titration profile of ferric Cc Thr12 ^{15}N amide resonance. The curves represent the best fit to a 1:1 binding model. The values of K_d and $\Delta\delta_0$ determined for wild type GALDH and its 9-fold surface mutant are 50 ± 10 µM and 0.75 ± 0.04 ppm, and 44 ± 10 µM and 0.93 ± 0.05 ppm respectively.

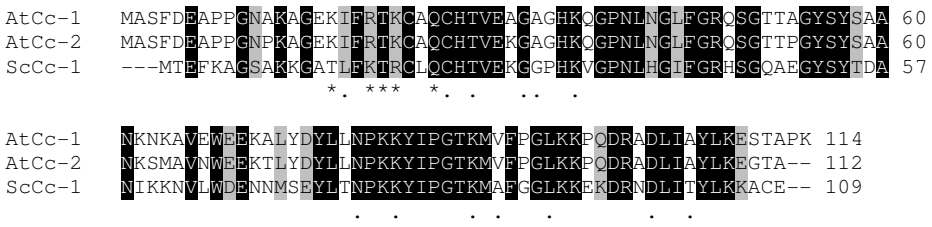

Figure 2.4. Clustal W multiple sequence alignment of both *Arabidopsis* Cc isozymes and yeast Cc (Sc*C*c-1). Accession numbers used are AtCc-1, At1g22840; AtCc-2, At4g10040; and ScCc-1, P00044. Identical residues are shaded in black, conservative mutations are shaded in grey. Residues of ScCc-1 showing the largest chemical shift perturbations (> 0.25 ppm) are marked with an asterisk (*) and residues showing moderate chemical shift perturbations (> 0.10 ppm) are marked with a dot.

Discussion

GALDH is one of the few members of the VAO-family that uses Cc as electron acceptor rather than molecular oxygen[65]. Related aldonolactone oxidoreductases are hydrogen peroxide producing oxidases that are unable to transfer electrons to Cc. Most aldonolactone oxidoreductases contain a covalently linked FAD[121-123]. Here, we studied the interaction of GALDH from *A. thaliana* with Cc in more detail and found that the interaction with Cc remains intact after removing 9 charged residues from the GALDH protein surface.

The interactions of GALDH and the 9-fold surface mutant were studied by NMR chemical shift perturbation analyses using ^{15}N-labelled ferric yeast Cc. The Cc residues involved in the interaction with GALDH are confined to a single surface patch on the front side of the molecule (Figure 2.2B). Only a few residues (Phe36 and Gly37 in case of the 9-fold surface mutant) on the back side are affected as well, which may represent transmitted effects due to a small movement of the haem group. In transient protein complexes, the size of chemical shift perturbations has been reported to correlate with the dynamics. Dynamic complexes show a small size of the chemical shift perturbations[29,124]. The chemical shift perturbations observed for the Cc – GALDH complex are small, suggesting that the complex is of a dynamic nature. For multiple, fast-exchanging, isoenergetic protein – protein configurations, the observed $\Delta\delta$ would be averaged over all orientations. This suggests that Cc and GALDH adopt different relative orientations within the complex, rather than form a single, well defined

structure. In such dynamic complexes, specific interactions, such as hydrogen bonds, and desolvation effects are likely to be minimal, also leading to small chemical shift perturbations. Recently, it was demonstrated that a similar complex, of Cc with adrenodoxin can be considered as a pure encounter complex[26].

Remarkably, wild-type GALDH and the 9-fold surface mutant show a similar binding constant for Cc. The binding map for the 9-fold surface mutant is very similar to wild-type GALDH, although there are subtle differences suggesting that in the complex with the 9-fold mutant Cc assumes a slightly less dynamic, more well-defined orientation relative to GALDH than in complex with wild-type GALDH. The removal of net 5 positive charges on the GALDH surface might result in less electrostatic repulsion between GALDH and Cc. Interestingly, binding maps of Cc in the complexes with GALDH (this work), cytochrome b_5 [124] and the non-physiological partner adrenodoxin[29] are strikingly similar. Moreover, chemical shift mapping studies of Cc in the complexes with bovine cytochrome b_5 [124], yeast cytochrome *c* peroxidase[125], cyanobacterial cytochrome *f* [126], pea plastocyanin[108], and GALDH (this work) indicate that Thr12 (Gln12 in horse heart C*c* used in ref.[108]) and Gln16 give the biggest binding shifts. This finding suggests that Cc employs a conserved set of surface-exposed residues, located around the haem edge, for the interactions with a variety of proteins (chapter 3). The residues of yeast Cc showing the highest chemical shift perturbations (> 0.25 ppm) are conserved among both *Arabidopsis* Cc paralogs, including Thr12 (Figure 2.4), suggesting that the interaction of GALDH and its physiological electron acceptors involves a similar mechanism.

In conclusion, it has been demonstrated that GALDH forms a transient and dynamic complex with Cc and the removal of 9 charged surface residues has little effect on the formation and dynamics of the complex indicating that a defined binding or docking site for Cc is absent from GALDH.