

Topological Analysis of CRALBP by Hydrogen/Deuterium Exchange and Mass Spectrometry

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OVERVIEW

- PURPOSE**
- Topological analysis of human CRALBP
- Identification of CRALBP ligand dependent conformational changes

- METHODS**
- Production of human CRALBP in *E. coli*
- Purification of apo CRALBP by nickel affinity chromatography
- Preparation of holo CRALBP with 11-*cis*-retinal
- Labeling of apo and holo CRALBP with deuterium
- LC-ESI/MS analysis of deuterium labeled intact CRALBP
- LC-ESI/MS analysis of peptide digests of deuterium labeled CRALBP

- RESULTS**
- Binding of 11-*cis*-retinal induces conformational changes in CRALBP structure.
- Amino and carboxy terminal domains (about 20-30 residues each) and residues 69-94 are all more solvent exposed in holo-CRALBP than in the apo form.
- CRALBP residues 198-212 and 224-243 are less solvent exposed in the holo protein and contain retinoid binding pocket components.

INTRODUCTION

The visual cycle is the enzymatic pathway by which all-*trans*-retinol from photoreceptor bleaching is isomerized to 11-*cis*-retinal in the retinal pigment epithelium for visual pigment regeneration. Cellular retinoid isomerase (CRALBP) serves as an acceptor of 11-*cis*-retinal in the isomerization step of visual cycle and as a modulator of 11-*cis*-retinoid dehydrogenase (Saari et al., 2001). Mutations in the CRALBP gene can cause progressive retinal degenerations leading to blindness. We are interested in the structure and function of CRALBP including protein-protein and protein-ligand interactions. Recently components of the CRALBP retinoid binding pocket have been identified using site-directed mutagenesis, chemical modification, NMR, UV-visible spectroscopy and fluorescence titration (Crabb et al., 1998a; Wu et al., 2001). CRALBP functions within a visual cycle protein complex in the retinal pigment epithelium and localization of sites of interaction are under investigation (Bhattacharya et al., 2002). Amide hydrogen-deuterium exchange (H/D exchange), particularly in combination with mass spectrometry, offers a powerful tool for studying interactions of proteins with ligands and for identifying solvent exposed regions that may interact with other proteins (Chang and Smith, 1993). Using HD exchange technology, we have initiated analysis of CRALBP topology and conformational changes associated with binding its endogenous ligand 11-*cis*-retinal.

METHODS

Purification of rCRALBP: Recombinant CRALBP was expressed in *E. coli* strain BL21 (DE3) lys5 grown in LB media (Crabb et al., 1998b). Bacterial cell pellets (3 g) were suspended in 9 ml lysis buffer (50 mM sodium phosphate buffer pH 7.8, 300 mM NaCl), incubated with DNase (2 μ g/ml/ml) at room temperature for 20-40 minutes and sonicated on ice for 5 minutes (20s on/10s off). The crude lysate was incubated with a nickel affinity support (1ml Ni-NTA resin) for 1 hour at 4°C and then poured into a small column. The column was washed with lysis buffer (2 L), lysis buffer containing 40 mM imidazole (1 L) and CRALBP was eluted with lysis buffer containing 250 mM imidazole. Protein amount was quantified by the Bradford method and by phenylthiocarbonyl amino acid analysis using Applied Biosystems model 420H, 120, 900 instrumentation.

Preparation of holo-CRALBP: Holo-CRALBP was prepared by incubating in the dark (4°C) 1.2 fold molar excess of 11-*cis*-retinal with purified apo-CRALBP (40 μ g, 1 mmol). Excess retinoid was removed with a Sephadex G25 spin column. Bleaching was by exposure to ambient light for 20 minutes. UV-visible spectroscopy was performed before and after bleaching with a Hewlett Packard 8453 diode array spectrophotometer.

LC-MS Peptide Mapping: To establish the pepsin fragmentation pattern of CRALBP, pepsin digestion of CRALBP (100 pmol) was performed with the same conditions that would be used to digest the deuterated apo and holo CRALBP in H/D exchange experiments. These conditions were 0°C, pH 2.5, a ratio of 2:1 by weight pepsin:CRALBP and a 5 min digestion time. These conditions minimized back exchange of deuterium. Following digestion, peptides were separated and analyzed by reversed phase HPLC directly coupled to the Micromass Q-ToF mass spectrometer (Miyagi et al., 2002). An Applied Biosystems Model 1400 HPLC system was used with a lysis C18 column (1 x 50 mm, 300Å, 5 μ m) and aqueous trifluoroacetic acid/acetonitrile solvents. The column, solvents and injector were maintained at 0°C to avoid back exchange. Peptides were eluted with a gradient of acetonitrile at a flow rate of 30 μ l per min. The mass spectrometer was calibrated with Glu fibrinopeptide B over a mass range of 100-2000 Da with a typical mass uncertainty of 10 ppm. Source and desolvation temperatures were maintained 60°C and 100°C respectively. Peptides were identified by matching their molecular masses with the calculated masses of CRALBP peptides derived from typical cleavage sites of pepsin (L, I, Y and W); peptide identities were confirmed by LC-MS/MS.

Hydrogen/Deuterium Exchange: HD exchange methods were essentially as described elsewhere (Wang et al., 2002). Aliquots of apo and holo CRALBP were incubated at room temperature in dark in D₂O buffer solution (Na₂HPO₄, D₂O, pH 6.9) for 5-60s. Exchange of hydrogens at the peptide amide linkages of a protein occurs within seconds at pH 6.9 if the hydrogen is exposed to the solvent and not involved in H-bonding, as it would be if it were in an α -helix or β -sheet. Isotope exchange was then quenched by lowering the pH to 2.5 and lowering the temperature to 0°C. Using Q-ToF2 and HPLC instrumentation, each sample was then analyzed by on-line LC-MS both as intact protein and as pepsin digests.

The masses of the peptides reflected the number of deuteriums that were incorporated into the intact protein during the D₂O incubation at pH 6.9, these results also reflected the exposure of that part of the protein to the solvent. Since the H/D exchange occurred before the pH was lowered and the protein was digested, incorporation of deuterium indicated which amide hydrogens in the complex were exposed to the solvent.

Figure 1

Methods used for topological analysis of CRALBP by hydrogen/deuterium exchange and mass spectrometry

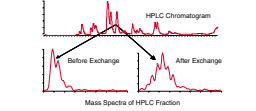
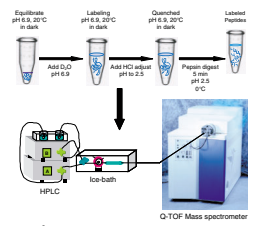


Figure 2

Human rCRALBP sequence and peptides identified following pepsin digestion

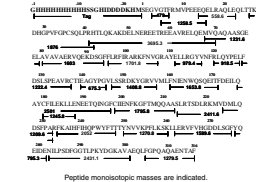


Figure 3

Decomvulted mass spectra of D₂O labeled intact CRALBP from early and late eluting chromatography fractions

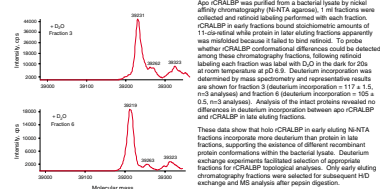


Figure 4

Optimization of CRALBP HD exchange conditions

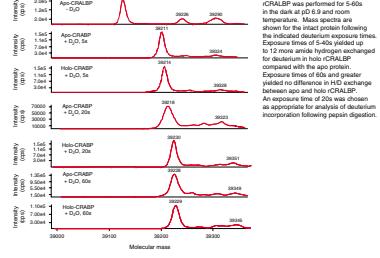


Figure 5

Total ion current of pepsin digests of apo and holo CRALBP

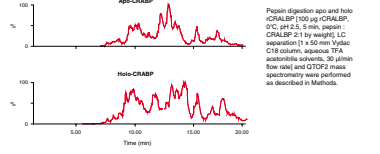
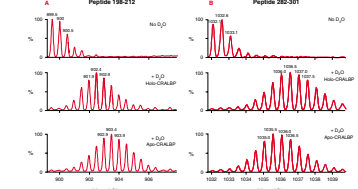


Figure 6

Examples of ligand dependent conformational changes



- A) Mass spectra for CRALBP peptide residues 198-212 are shown before (No D₂O) and after deuterium labeling of holo and apo CRALBP. More deuterium was incorporated into CRALBP containing bound 11-*cis*-retinal.
- B) Mass spectra for CRALBP peptide residues 220-301 are shown before (No D₂O) and after deuterium labeling of holo and apo CRALBP. More deuterium was incorporated into this peptide than apo CRALBP, indicating that this region is more solvent accessible in CRALBP containing bound 11-*cis*-retinal.

Figure 7

Comparison of HD exchange in apo and holo CRALBP

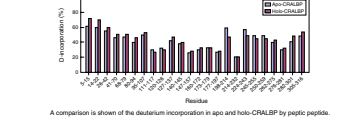


Figure 8

Summary of the differences in HD exchange in apo and holo CRALBP

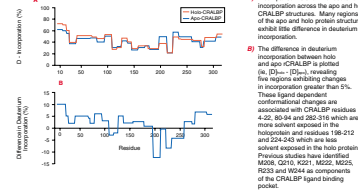
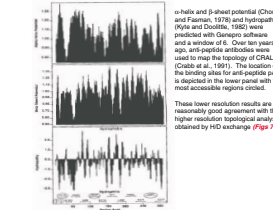


Figure 9

Secondary structure and hydrophathy profiles for bovine CRALBP



CONCLUSIONS

- Early eluting nickel affinity chromatography fractions contain properly folded rCRALBP based on retinoid binding properties. HD exchange analysis support differences in CRALBP conformations in early and late eluting fractions.
- Binding of 11-*cis*-retinal induces conformational changes in the rCRALBP structure detectable by HD exchange mass spectrometry.
- Ligand dependent conformational changes were observed in rCRALBP residues 42-94 and 282-316 which are more solvent exposed in the holo protein.
- Ligand dependent conformational changes were also observed in rCRALBP residues 198-212 and 224-243 which are less solvent exposed in the holo protein. Previous studies have shown that this region contains ligand binding pocket components.

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