Photoaffinity Labeling of the CRALBP Ligand Binding Pocket

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Overview

Purpose

- To characterize the structure of the ligand binding pocket in the cellular retinaldehyde-binding protein (CRALBP)

Methods

- Photoaffinity labeling with 3-diazo-4-keto-11-*cis*-retinal
- UV-visible spectroscopy
- Chemical reduction of protein bound retinal with $NaB[^3H]_4$
- Alkylation of cysteine with iodoacetamide
- Protein quantification by amino acid analysis
- Radioactivity quantification by scintillating counting
- RP HPLC
- Peptide characterization by MALDI TOF MS and LC MS/MS

Results

- Eight photoaffinity modified amino acid residues have been identified in CRALBP
- M208, K221, M222, and M225 were modified and are previously established CRALBP retinoid binding pocket residues
- Y179, F197, C198 and V223 were also modified and may represent newly identified CRALBP ligand binding pocket components

Introduction

The visual cycle is the enzymatic pathway by which all-*trans*-retinal from photoreceptor bleaching is isomerized to 11-*cis*-retinal in the retinal pigment epithelium (RPE) for visual pigment regeneration. In the RPE, CRALBP (cellular retinaldehyde-binding protein) serves as an 11-*cis*-retinol acceptor and as a modulator of 11-*cis*-retinol dehydrogenase in the mammalian rod visual cycle (Saari et al., 2001). Recently we have determined that CRALBP interacts with a number of other proteins in a visual cycle protein complex (Bhattacharya et al., 2002). Mutations in the CRALBP gene can cause progressive retinal degenerations that lead to blindness. In the retina, CRALBP carries light sensitive 11-*cis*-retinal and 11-*cis*-retinol as endogenous, noncovalent ligands. Structure-function studies in our laboratory have identified several residues in the CRALBP retinoid-binding pocket using chemical modification, site-directed mutagenesis, UV-visible spectroscopy, fluorescent titration and NMR (Crabb et al., 1998a; Wu et al., 2001). To further characterize the structure of the CRALBP retinoid-binding pocket and better understand CRALBP ligand interactions, we have utilized photoaffinity labeling with the retinoid analogue 3-diazo-4-keto-11-*cis*-retinal (Borhan et al., 2000).

Methods

Preparation of rCRALBP. Human recombinant CRALBP was expressed in *E. coli* strain BL21 (DE3) LysS with the pET 19b vector and the apoprotein purified to apparent homogeneity by nickel affinity chromatography (Crabb et al., 1998b).

Retinoid Labeling of rCRALBP. Purified apo rCRALBP (in 50 mM Tris-HCl, pH 7.0, 1mM DTT-EDTA) was incubated with 3-diazo-4-keto-11-*cis*-retinal (DK-11-*cis*-retinal) (Borhan et al., 2000) or 11-*cis*-retinal (1.5 fold excess relative to rCRALBP) at 4°C in the dark for an hour. Unbound retinoid was removed by Sephadex G25 spin column chromatography. Spectra of holo-rCRALBP were measured by scanning UV-visible spectroscopy (HP 8453 A diode array UV visible spectrometer) from 250 nm to 500 nm. Bleaching (photoisomerization of 11-*cis*- to all-*trans*-retinal) was by exposure of holo rCRALBP to ambient light for 20 minutes.

Photolysis Conditions. rCRALBP complexed with DK-11-*cis*-retinal was exposed to UV irradiation at 254 nm at -80°C in the dark from 5s to 20 min in 50 mM Tris-HCL, pH 7.0, 1mM DTT-EDTA containing 67% glycerol. The distance between the UV lamp and sample was 2 cm and the output of the lamp was 18.4W. Following photolysis, unreacted DK-11-*cis*-retinal was extracted with hexane (2:1, v/v). Glycerol was removed by Centricon buffer exchange into 50 mM Tris-HCL, pH 7.0, 1mM DTT-EDTA and the sample dried in a speedvac.

Radioactive Labeling of rCRALBP. Photoaffinity modified rCRALBP was resuspended in ultrapure, deionized 8M urea and the covalently attached retinal reduced to retinol by incubating with tritiated $NaB[^3H]_4$ (5:1 molar ratio) at room temperature for 15 minutes followed by addition of cold $NaBH_4$ (10mg) in 0.1 M NaOH (1µl). The ^3H-labeled rCRALBP was dialyzed exhaustively against 0.1% TFA to desalt and remove unbound tritium.

Quantification of Modified rCRALBP. Tritiated photoaffinity modified rCRALBP was quantified by PTC amino acid analysis using an Applied Biosystems model 420H/130/920 automated analysis system (Crabb et al., 1997). Radioactivity was quantified by scintillation counting.

Alkylation of Cysteine. For alkylation of Cys, tritiated photoaffinity labeled rCRALBP was incubated in 8 M urea, 100 mM Tris-HCL, pH 8.0, 10 mM DTT under argon for 10 minutes at room temperature. Iodoacetamide (1.2 fold over total SH content) was added and incubated at room temperature for another 15 minutes, then 1/3 more IAN was added and the incubation continued for another 15 minutes. The reaction was stopped by addition of a 2 fold molar excess of DTT over iodoacetamide.

Tryptic Digestion and RP HPLC. Carboxyamidomethyl, tritiated, photoaffinity modified rCRALBP was digested overnight at 37°C with sequence grade trypsin (20:1, Wt/Wt, Promega). Tryptic digests were separated by RP-HPLC using Applied Biosystems model 120A HPLC system, a 5 µ Vydac C18 column (2.1 x 150 mm), aqueous trifluoroacetic acid/acetonitrile solvents and a flow rate of 100 µl/min. Fractions were collected for mass spectrometric analysis and scintillation counting.

Mass Spectrometry. All RP HPLC fractions of the tryptic digest were analyzed by MALDI-TOF MS using a Voyager DE Pro instrument (Applied Biosystems) (West et al., 2001) and by LC MS/MS (Miyagi et al., 2002) using a QTOF2 instrument equipped with a CapLC (Micromass). LC MS/MS was performed with a New Objective C18 column (75 µ x 5 cm), aqueous formic acid/acetonitrile solvents and using a pump rate of 8 µl/min and a precolumn splitter (30:1), yielding a flow rate of 250 nl/min at the column. The electrospray mass spectrometric analyses were performed in the positive ion mode with a source temperature of 100°C and 2.5 kV potential. The cone voltage was 35 V and collision energy was 30 V. The MS/MS data were analyzed with Masslynx 3.5 software (Micromass).

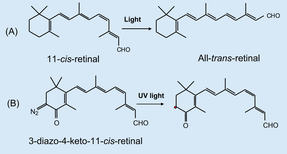

Figure 1. Photoaffinity Labeling with a Light Sensitive Retinoid

(A) The structures of 11-*cis*-retinal and all-*trans*-retinal. Light isomerizes the 11-*cis* bond to the all-*trans* configuration, dislodging the ligand from the rCRALBP retinoid-binding pocket.
(B) Photoaffinity labeling with DK-11-*cis*-retinal. UV light replaces the diazo group in with a free radical which can then move freely throughout the conjugated double bond structure and insert itself in vicinal polypeptide for stabilization. Since photoisomerization of the 11-*cis* bond will destroy rCRALBP binding specificity, photolysis conditions were sought that minimized nonspecific insertion.

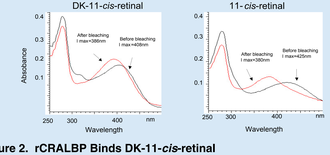

Figure 2. rCRALBP Binds DK-11-*cis*-retinal

UV-visible spectra are shown for rCRALBP complexed with DK-11-*cis*-retinal and 11-*cis*-retinal. rCRALBP with bound 11-*cis*-retinal exhibits a characteristic chromophore maximum at 425 nm and bound with DK-11-*cis*-retinal a maximum at 408 nm. Upon bleaching, these maxima shift to ~380 nm due to the production of free all-*trans* retinoids.

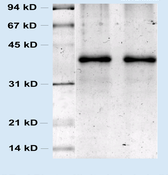

Figure 3. SDS-PAGE of Photoaffinity Labeled rCRALBP

Coomassie blue stained SDS-PAGE of rCRALBP (1 µg) is shown before and after photoaffinity labeling with DK-11-*cis*-retinal.

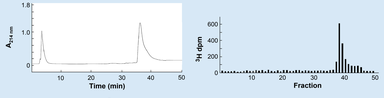

Figure 4. RPHPLC of ^3H-Photoaffinity Labeled-rCRALBP

The RP-HPLC absorbance and radioactivity profiles are shown for photoaffinity labeled rCRALBP (1 µg, 25.6 pmol) following treatment with tritiated $NaB[^3H]_4$. RP HPLC was performed with a 5µ Vydac C18 column (1 x 150 mm), aqueous TFA/acetonitrile solvents, gradient elution, a flow rate of 50 µl/min and 1 min fractions were collected. These results support the presence of a covalent radioactive label on rCRALBP.

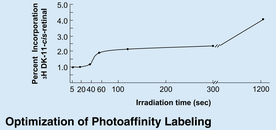

Figure 5. Optimization of Photoaffinity Labeling

Photolysis of rCRALBP with bound DK-11-*cis*-retinal was performed for 5s to 20 min and the amount of covalent incorporation determined following reduction with $NaB[^3H]_4$ by scintillation counting and amino acid analysis. Long (20 min) irradiation times yielded about 4% incorporation but modifications were detected throughout the protein structure by LC MS/MS. Short (5-40s) irradiation times yielded relatively constant incorporation levels (~1%) therefore 5s photolysis times were chosen to minimize nonspecific protein modifications.

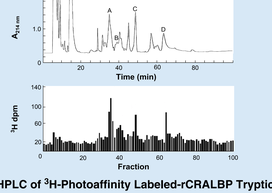

Figure 6. RPHPLC of ^3H-Photoaffinity Labeled-rCRALBP Tryptic Digest

The RP-HPLC absorbance and radioactivity profiles are shown for ^3H-photoaffinity labeled-rCRALBP (195 µg, 5 nmol) following digestion with trypsin. RP HPLC was performed with a 5µ Vydac C18 column (2.1 x 150 mm), aqueous TFA/acetonitrile solvents, gradient elution, a flow rate of 100 µl/min and 1 min fractions were collected. Radioactive fractions 35-37, 39-41, 49-51 and 64-66 were pooled and designated A, B, C, D, respectively. The total recovery of radioactivity was ~60 % of the amount applied.

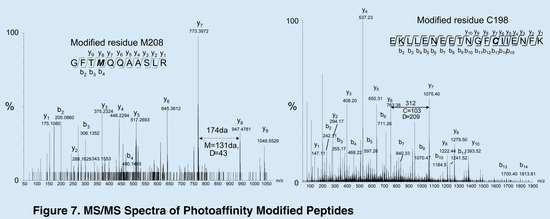

Figure 7. MS/MS Spectra of Photoaffinity Modified Peptides

MS/MS spectra from two photoaffinity modified peptides are shown as examples. Radioactive fraction A was found to contain rCRALBP residues 205-215 and M208 was identified with a mass addition of 43 Dalton. Radioactive fraction D was found to contain rCRALBP residues 186-204 and C198 was identified with a mass addition of 209 Daltons. A total of eight photoaffinity modified residues were identified as summarized in Table 1, all with different mass additions. Variability in the observed modification mass may be due to free radical migration throughout the conjugated double bonds of the retinoid ligand.

Table I. Photoaffinity Modified rCRALBP Residues Identified by LC MS/MS

Fraction	Modified Residue	Sequence	Mass of Modification
B	***Y***179	***Y***CFILEK	46
B	***F***197	***F***CIIENFK	62
D	***C***198	LLENEETQINGF***C***IIENFK	209
A	***M***208	GFT***M***QQAASLR	43
C	***K***221	***K***MVDMLQDSFPAR	74
B,C	***M***222	***M***VDMLQDSFPAR	110, 43
C	***V***223	***V***DMLQDSFPAR	99, 115
C	***M***225	KMVD***M***LQDSFPAR	32

Photoaffinity modified residues are indicated in bold and italics.

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SEGVGTFRMVPEEEQELRAQLEQLTTKDHGPVFGPCSQLPRHTLQKAKDE 50

LNEEETREEAVRELQEMVQAQAASGEELAVAVAERVQEKDSGFFLRFIR 100

ARKFNVGRAYELLRGYVNFRLQYPELFDSLSPEAVRCTIEAGYPGVLSSR 150
          165                 179                 197
DKYGRVVMLFNIEMMQSQEITFDEILQAYCFILEKLLENEETQINGFCIIE 200
                                                198
   208 210           221 223225        233       244
ENFKGFTMQQAASLRTSDLRKMVDMLQDSFPARFKAIHFIHQMWYFTTTY 250
                    222
NVVKPFLKSKLLERVFVHGDDLSGFYQEIDENILPSDFGGTLPKYDGKAV 300

AEQLFGPQAQAENTAF
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Figure 8. rCRALBP retinoid-binding pocket components

The sequence of human CRALBP is shown with photoaffinity labeled residues identified in this study shown in blue and green. Residues in blue and red have previously been established as retinoid binding components (Crabb et al., 1998; Wu et al., 2001). Residues in green are newly identified possible components of the ligand binding pocket.

Table II. Summary of Unmodified rCRALBP Peptides Identified by LC MS/MS[a]

Residues	Mass calc.	Mass obs.	Mass Error	HPLC Fraction	Sequence
1-31	3616.56	3616.58	0.02	35-40	GHHHHHHHHHHSSGHIDDDDKHMSEGVGTFR
22-31	1119.52	1119.51	0.01	35-37	HMSEGVGTFR
32-41	1258.59	1258.59	0.00	14-19	MVPEEEQELR
34-41	1028.49	1028.49	0.00	14	PEEEQELR
42-50	1030.57	1030.57	0.00	32-38	AQLEQLTTK
43-50	959.56	959.56	0.00	34	QLEQLTTK
51-64	1565.73	1565.74	0.01	39-41	DHGPVFGPC[b]SQLPR
65-69	625.34	625.60	0.28	15-16	HTLQK
70-77	1488.72	1488.78	0.06	7-9	AKDELNEREETR
70-86	2073.02	2073.09	0.07	12	AKDELNEREETREEAVR
72-81	1289.59	1289.64	0.05	11-19	DELNEREETR
72-86	1873.88	1873.95	0.07	11	DELNEREETREEAVR
87-109	2428.20	2428.22	0.02	62-67	ELQEMVQAQAASGEELAVAVAER
98-109	1229.64	1229.64	0.00	30-32	SGEELAVAVAER
96-109	1371.75	1371.71	0.04	30	AASGEELAVAVAER
94-109	1568.73	1568.7	0.03	40	AQAASGEELAVAVAER
110-120	1324.69	1324.68	0.01	46	VQEKDSGFFLR
114-120	840.42	840.41	0.01	46-47	DSGFFLR
121-123	434.27	434.30	0.03	47	FIR
124-125	373.25	373.24	0.01	6-7	ARK
126-131	719.42	719.43	0.01	47	KFNVGR
132-143	1499.80	1499.79	0.01	30	AYELLRGYVNFR
144-159	1862.95	1862.94	0.01	58-68	LQYPELFDSLSPEAVR
149-159	1232.69	1232.69	0.00	62-63	LFDSLSPEAVR
151-159	972.48	972.52	0.04	60-61	DSLSPEAVR
160-173	1508.72	1508.71	0.01	40-48	C[b]TIEAGYPGVLSSR
160-173	1491.72	1491.72	0.00	40-48	C[b]TIEAGYPGVLSSR
161-173	1348.72	1348.69	0.03	40	TIEAGYPGVLSSR
174-178	637.33	637.40	0.07	9-10	DKYGR
179-208	3720.81	3720.83	0.02	91-93	VVMLFNIENWQSQEITFDEILQAYC[b]FILEK
209-227	2310.11	2310.16	0.05	62-63	LLENEETQINGFCIIENFK
228-238	1208.61	1208.64	0.04	48-52	GFTMQQAASLR
228-238	1224.61	1208.62	0.01	48-52	GFTMQQAASLR
239-244	718.41	718.40	0.01	12-13	TSDLRK
244-256	1536.75	1536.74	0.01	48-52	KMVDMLQDSFPAR
244-256	1552.75	1552.82	0.07	48-52	KMVDMLQDSFPAR
245-256	1408.66	1408.65	0.01	48-52	MVDMLQDSFPAR
245-256	1424.66	1424.68	0.02	48-52	MVDMLQDSFPAR
245-256	1440.66	1440.7	0.04	48-52	MVDMLQDSFPAR
245-258	1654.82	1654.85	0.03	57	MVDMLQDSFPARFK
257-281	3124.68	3124.67	0.01	70	FKAIHFIHQPWYFTTTYNVVKPFLK
263-287	2140.08	2140.03	0.05	64-68	IHQPWYFTTTYNVVKPFLKSKLLER
259-281	2849.51	2849.51	0.00	67-68	AIHFIHQPWYFTTTYNVVKPFLK
288-317	3308.60	3308.60	0.00	65-75	VFVHGDDLSGFYQEIDENILPSDFGGTLPK
318-321	481.23	481.20	0.03	7-9	YDGK
322-339	1890.92	1890.93	0.01	50-64	AVAEQLFGPQAQAENTA

a. LC MS/MS analyses of photolyzed DK-11-cis-retinal rCRALBP have identified 100% of the protein sequence. All photoaffinity modified residues are listed in table I.
b. Carboxyamidomethyl cysteine

Conclusions

- Following photoaffinity labeling with DK-11-*cis*-retinal, peptides accounting for 100% of the rCRALBP sequence were recovered and eight modified amino acids identified.
- CRALBP residues M208, K221, M222, and M225 were identified as photoaffinity modified and are part of the retinoid binding pocket based upon previous site-directed mutagenesis studies.
- Y179, F197, C198 and V223 were also photoaffinity modified and likely are part of the CRALBP ligand binding pocket. The functional significance of these residues is under investigation.

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