Relaxation of β-Structure in Tear Lipocalin and Enhancement of Retinoid Binding

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PURPOSE. To study binding of retinoids to human tear lipocalin (TL) to assess factors influencing ligand affinity and delivery. Mechanistic features of retinoid interactions with TL were investigated, including the influence of the retinoid functional group on ligand affinity, the relative affinity of retinol versus fatty acids, the influence of relaxation of secondary structure in TL on ligand binding, the role of specific conserved hydrophobic residues in maintaining the rigidity of the secondary structure, and the potential release of retinol in a low-pH environment that promotes structural relaxation at lipid interfaces.

METHODS. The binding and displacement of retinoids were monitored by quenching of protein fluorescence. Circular dichroic spectra were used to evaluate structural and conformational changes in TL-retinoid complexes. Site-directed mutagenesis was performed to determine the influence of the residues Trp17, Ile98, Gly15, and Leu19 in retinoid binding to TL and to correlate these effects with changes in secondary structure.

RESULTS. Retinal and retinol bound TL with similar affinity. Fatty acids competed with retinoids for the same binding site on TL. Optical activity associated with retinal binding to TL was reduced in the presence of palmitic acid. In comparison with TL, the mutants W17C and J98C displayed relaxation of secondary structure, manifested as diminution of β-sheet content in conjunction with a destabilization in urea, reduced aromatic asymmetry, and greater binding affinity for retinoids. Unlike fatty acids, retinol is not released from TL at low pH.

CONCLUSIONS. The unique spectral properties of retinoids permit the simultaneous study of structural changes in TL and ligand binding. Retinoid binding is enhanced by specific mutations that induce relaxation of TL structure but is altered minimally by the functional group in retinoids. Two key hydrophobic residues, Trp17 (A strand) and Ile98 (G strand), contribute to backbone rigidity and influence retinoid binding through their participation in an internal hydrophobic cluster and external hydrophobic patch, respectively. The contributions of these sites to ligand binding may explain their conserved nature in the lipocalin family. Information regarding the binding and release of retinoids compared with fatty acids favors a role for TL in the delivery of lipids other than retinol to the tear film interfaces. (Invest Ophthalmol Vis Sci. 2002;43:3165-3173)

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ment of Ile98 with other amino acids is likely to result in detectable conformational and structural alterations. Residues Trp17 and Ile98 were chosen for site-directed mutagenesis to search for structural changes in TL with the binding of retinoids. Because the magnitude of optical activity of retinoids bound to TL correlates with binding.12,17 Circular dichroic (CD) spectroscopy permitted simultaneous monitoring of retinoid binding and the associated structural changes in TL.

Information regarding retinoid binding in other family members, such as β-lactoglobulin and RBP, may have relevance for TL. In both β-lactoglobulin and RBP, retinoids occupy the cavity with a defined orientation so that the β-ionone ring is oriented toward the calyx and the alcohol, aldehyde, or acid group is oriented toward the lipocalin mouth.24 Bound bound to RBP exhibits optical activity of the chromophore absorption band.25 Limited information is available regarding the binding site and orientation of retinol in binding to TL. The holo-TL-retinol complex shows diminished optical activity in the region of 300 to 340 nm compared with the spectrum of the apo-TL-retinol complex. The decreased rotational strength may reflect a lower affinity of retinol than other lipids,12,14 but a direct comparison is needed, particularly with lipids that are abundant in tears. The relative affinity of retinol versus fatty acids for TL and the characterization of competition for binding sites may provide insight into the carrier function of TL.

The unique spectral properties of retinoids and broad promiscuity of TL provide an opportunity to compare fatty acid binding with retinol binding to TL and further to study the changes in secondary structure that occur with ligand binding. The delineation of specific regions in TL that influence ligand specificity by conferring structural rigidity may have important functional implications for other members of the lipocalin family.

MATERIALS AND METHODS

Reagents

All trans-retinal, all trans-retinol, lauric acid (C12), myristic acid (C14), and palmitic acid (C16) were purchased from Sigma Chemical Co. (St. Louis, MO). Reagents and solvents were of analytical grade or better. Escherichia coli BL21 (DE3) cells were obtained from Novagen, Inc. (Madison, WI). Oligonucleotide primers were obtained from GibcoBRL Life Technologies, Inc. (Grand Island, NY).

Spectrophotometry

Apo- and holo-TL were incubated with retinal (1:2 molar ratio) and the mixture was passed through a gel-filtration column (Sephadex G-25; Amersham Pharmacia Biotech, Piscataway, NJ) to remove free retinal. Absorbance measurements were made at 20°C in a spectrophotometer (model UV-2401PC; Shimadzu Instruments, Inc., Columbia, MD).

Spectrofluorimetry

Fluorescence measurements were made at 20°C in a spectrofluorometer (Spectro Fluorolog-3; Jobin Yvon, Edison, NJ). The bandwidth for excitation and emission was 2 nm. Raman and background scattering by the solvent were corrected when necessary with the appropriate blank solutions. Binding of retinal and retinol were evaluated by observing the quenching of protein fluorescence, as previously described.20 Excitation and emission wavelengths were set at 295 and 334 nm, respectively. For a blank, N-acetylated-tryptophanamide solution with fluorescence equal to that of the protein at excitation wavelength of 295 nm was used, as previously described.20 The binding data were analyzed according to Cogas et al.20 Protein concentrations, ranged from 1.5 to 2.1 μM. Sodium phosphate (10 mM) and sodium citrate buffer (30 mM) were used for pH-dependent experiments at pH 7.3 and 3.0, respectively.

For competition experiments, progressively increasing amounts of fatty acids dissolved in ethanol were added to the apo-TL-retinol complex (2.5 μM apo-TL and 2 μM retinol) at pH 7.3 or 3.0. The final ethanol concentration did not exceed 2%.

Circular Dichroic Spectroscopy

Spectra were recorded (600 spectropolarimeter; Jasco, Inc., Easton, MD; 0.2 mm path length for far-UV spectra and 10-mm path length for near-UV spectra) using a protein concentration of 1.2 mg/mL in 10 mM sodium phosphate (pH 7.5). Eight or 16 scans each from 190 to 260 nm and 250 to 320 nm were averaged, respectively. Results were recorded in millidegrees. For the far-UV CD spectra, the units were converted to mean residue molar ellipticity. Computer-assisted estimations of α-helix and β-sheet content in TL were made by the self-consistent method, including the Hennessey-Johnson, Karplus-Sandrock, and Levitt-Greer formulas.27

Urea-unfolding experiments were performed as previously described.18 CD spectra were obtained at 25°C, and the fractions of unfolded proteins were plotted as the fractional changes in ellipticity (θ) at 218 nm versus progressively increasing concentration of urea.18

Site-Directed Mutagenesis and Plasmid Construction

The TL cDNA in PCR II (Invitrogen, San Diego, CA), previously synthesized,28 was used as a template to clone the TL gene-spanning bases 115 to 592 of the previously published sequence29 into pET 20b (Novagen, Inc., Madison, WI). Flanking restriction sites for Ndel and BamHI were added to produce the native protein sequence as found in tears.29 We prepared the TL mutants I98C, W17C, W17F, W17Y, G15C, and L19C with oligonucleotides (Life Technologies, Inc.) using the previously published method of introduction of a point mutation by sequential PCR steps.29 Amino acid 98 corresponds to the Ile, bases 406 to 408, of Redl et al.5

Expression and Purification of Mutant Proteins

The mutant plasmids were transformed in E. coli BL 21 (DE3) cells, and cells were cultured and the protein expressed according to the manufacturer’s protocol (Novagen, Inc.). After cell lysis as previously described,51 the supernatant was treated with methanol (40% final concentration) at 4°C for 2.5 hours. The suspension was centrifuged at 3000g for 30 minutes. The supernatant was dialyzed against 50 mM Tris-HCl (pH 8.4). The dialysate was treated with ammonium sulfate at 45% to 75% saturation. The resultant precipitate was dissolved in 50 mM Tris-HCl (pH 8.4) and applied to a gel-filtration column (2.5 × 100 cm; Sephadex G-100; Amersham Pharmacia Biotech, Inc.) equilibrated with 50 mM Tris-HCl and 100 mM NaCl (pH 8.4). The fraction containing the mutant protein was dialyzed against 50 mM Tris-HCl (pH 8.4) and applied to an anion exchange column (DEAE-Sephadex A-25; Amersham Pharmacia Biotech, Inc.). Bound protein was eluted with a 0- to 0.8 M NaCl gradient. Eluted fractions containing mutant proteins were centrifugally concentrated (Centricon-10; Millipore Corp. Bedford, MA). The purity of mutant proteins was verified by SDS tricine gel electrophoresis.1 Protein concentrations were determined by the biuret method.32 Delipidation was performed by chloroform-methanol extraction.1

RESULTS

The absorbance spectra of apo- and holo-TL incubated with retinal both showed absorbance at approximately 370 nm after size-exclusion chromatography (Fig. 1). The peaks in this region were due to the absorbance of bound retinal. The retinal absorption band in holo-TL had multiple small peaks, and the intensity was lower than the peak for apo-TL. These features suggest that native lipids on holo-TL interfere with retinal binding and/or alter the environment of retinal.
TL contains a single tryptophan (Trp17) that exhibits fluorescence at a maximum wavelength of 334 nm.\textsuperscript{15} It is evident from Figure 2A that the relative fluorescence of the tryptophan in apo-TL may have been quenched by incubation with retinal. After correction for the nonspecific fluorescent quenching of free retinal, the Cogan plot (Fig. 2B) revealed a dissociation constant ($K_d$) of 0.39 $\mu$M. These results may be compared with those obtained with retinol, in which $K_d = 0.19$ $\mu$M (Figs. 2C, 2D). Retinol had binding affinities similar to those of apo-TL at both pH 7.3 and 3.0 ($K_d = 0.13$ $\mu$M, respectively).

To determine whether retinoids bind to the same site on TL as fatty acids, competitive binding experiments were performed and are summarized in Table 1. Fatty acids displaced retinol, implying that there is a single binding site in TL for both ligands (Fig. 3). Also, fatty acids with longer alkyl chains displaced retinol more effectively (Fig. 3). The effect of low pH on the effective displacement of retinol by fatty acids was apparent (Fig. 3). At pH 3.0 there was less displacement of retinol by fatty acids than at pH 7.3. Further, the retinol-binding affinity at pH 7.3 was found to be similar to that at pH 3.0. Therefore, decreased displacement of retinol by fatty acids at pH 3.0 may be attributed to the decreased binding affinity of TL for fatty acids, evident in Figure 2 and in previous work.\textsuperscript{15}

It is evident from near-UV CD spectra that the binding of retinal to TL can be monitored by the optical activity of the chromophore absorption band with minima in the range of 368 to 377 nm (Fig. 4). Comparison of the CD spectra for apo- and holo-TL reveals that the optical activity of retinal was at least two times greater when combined with apo-TL than with holo-TL (Fig. 4). The greater optical activity reflects greater binding affinity for apo-TL.\textsuperscript{12,15} Retinal did not completely displace native ligands in holo-TL. The influence of the native tryptophan was studied by comparison of TL with the tryptophanless mutants W17F and W17Y. The CD spectra for the mutant proteins showed loss of optical activity in the aromatic region. As expected, the peak associated with the absorption of tryptophan at 290 nm was absent. Mutants W17F and W17Y replaced tryptophan with other aromatic residues and showed decreased optical activity in the region that is associated with binding of retinal (320–400 nm) when compared with holo- and apo-TL. In addition, the CD spectra of both mutants showed minima at 391 and 357 nm, respectively, and a relatively positive contribution to optical activity in the region of 300 to 320 nm (Fig. 4). These changes reflect an altered conformational state of retinal in the retinal-TL complex con...
taining the aromatic residue replacement mutants, compared with the environment with tryptophan in place.

The tryptophan substitution W17C replaces the hydrophobic aromatic residue with a nonaromatic residue. The influence on retinal binding is evident in Figure 5. Markedly increased optical activity (increased approximately sevenfold) with a positive peak at 402 nm reflects induced asymmetry and tighter retinal binding. There was much less effect with a neighboring substitution, G15C, but the spectrum still showed a positive contribution from retinal in the 400-nm region (Fig. 5). For substitution L19C, the spectrum had a different shape, with both positive and negative peaks (Fig. 5). The optical activity in the 400-nm region in I98C was enhanced to a greater degree than in G15C or L19C but to a lesser degree and with an opposite sign than in W17C. These data are evidence that the conserved Trp17, as well as Ile98, may be important in modulating the binding of retinal and that amino acid substitutions in these regions affect the binding of retinal. Significant changes in magnitude and in the sign of optical activity in the CD spectra show that substitutions of the conserved tryptophan change the conformational state of bound retinal. The neighboring substitutions G15C and L19C had a similar but less dramatic effect.

Figure 6 shows the effect of displacement of retinal with palmitic acid. In the region of 368 nm, the optical activity associated with the binding of retinal was markedly reduced in the presence of palmitic acid. Corroborating these results, palmitic acid displaced retinal from the retinal–TL complex, as indicated by diminished quenching of the Trp fluorescence of TL (data not shown).

Changes in aromatic side-chain asymmetry were manifest with the amino acid substitutions at or near residue 17 and at position 98 of TL in the near-UV CD spectra (Fig. 7). Compared with holo-TL, all the mutants showed less optical activity in the aromatic regions. Mutants I98C and W17C showed the most dramatic loss in aromatic asymmetry. In addition, W17C did not display optical activity at 290 nm; the Lb band of tryptophan was absent. This substitution removed the sole tryptophan in TL.

The influence of mutagenesis on retinol binding is apparent in Figure 8 and was similar to the results for retinal binding.
The binding of retinol to the mutant W17C yields increased optical activity in the region of 350 nm, reflecting more avid binding. With mutants W17Y and W17F, the changes were less in amplitude.

The contribution of Trp17 and Ile98 to the secondary structure of TL was evident from the far-UV CD spectra of the substitutions W17C, G15C, L19C, and I98C (Fig. 9, Table 2). Substituted mutants, W17C and I98C, featured shifted minima in the region of 205 to 208 nm, indicative of an increase in random coil and a corresponding decrease in β-sheet structure. Mutants G15C and L19C revealed minima (approximately 214 nm) similar to that of TL. Mutants that exhibit reduction of β structure, reduction in aromatic side-chain asymmetry, and conformational alterations also have increased binding affinity for retinol. Reduced β structure was also manifested in destabilization of the protein in urea. G15C and L19C showed some reduction in the threshold for denaturation in urea but less than W17C.18 Mutant I98C showed the most destabilization compared with TL and the other mutants (Fig. 10). The addition of retinol increased the midpoint of transition (Fig. 10).

DISCUSSION

Members of the lipocalin family have a wide range of disparate functions, despite a similar overall structure.14 Knowledge of specific structural features that modulate the binding of ligands is essential for understanding how each member performs its unique functions. The importance of Trp17 and Ile98 in the secondary structure of lipocalin was clearly demonstrated in these experiments. Site-directed mutagenesis that replaced an aromatic residue with a nonaromatic residue at position 17 resulted in loss of β structure and an increase in random coil formation (Fig. 9). The effect was less pronounced for mutations G15C and L19C. Positions 17 and 19 are part of the A strand of TL, but position 15 is an exposed residue.21 Both Gly15 and Trp17 are highly conserved in the lipocalin family. The more pronounced changes observed with a mutation of Trp17 may reflect a susceptible position facing internally at the tip of the cavity. That only minor alterations occurred with G15C verifies that the structure of TL is exquisitely sensitive to changes at key positions rather than to the overall composition.
of residues. As a strand with β structure, the residues alternate their positions in relation to the cavity so that residues 17 and 19 face internally in the cavity.21 In addition to the loss in β structure, there was a corresponding decrease in aromatic side-chain asymmetry and greater binding of retinal with these mutations, particularly of W17C. The more permissive structure permitted more avid binding of retinoids to TL. In contrast, the mutants W17F and W17Y, both aromatic substitutions, showed a protein with retained β structure18 and less retinoid binding (Figs. 4, 8). The relationship between protein structure and retinoid binding is also evident from substitution I98C that produced a protein with decreased structure, greater retinal binding, and an altered conformational state of the retinal-TL complex. Our data show that there was increased retinol binding to those TL mutants that exhibited relaxation of secondary structure (Figs. 8, 9). There was accompanying increased urea-induced instability of the I98C mutant protein (Fig. 10) compared with other mutants and TL. Reduction of the conserved disulfide bond of TL also results in relaxation of structure and greater binding of retinoids.12,17 Taken together, these findings support the concept that the features that confer a rigid structure also exert a restrictive influence on retinoid binding in TL.

It is intriguing to speculate that the relaxation of structure in TL, associated with nonaromatic substitutions at positions 17 and 98, results from disruption of interactions among clusters of hydrophobic residues. The analogous residues in β-lactoglobulin, Trp19 and Tyr102, are in close proximity (Trp19 [CZ3]-Tyr102 [CB] = 0.49 nm [PDB ID:1B0O; the Protein Data Bank is hosted by the Research Collaboratory for Structural Bioinformatics, a consortium, and is available in the public

Table 2. Estimation of α-Helix and β-Sheet Content in Wild-Type TL and Mutants

<table>
<thead>
<tr>
<th>Submethod</th>
<th>WT-TL*</th>
<th>W17C*</th>
<th>G15C</th>
<th>L19C</th>
<th>I98C</th>
<th>W17Y*</th>
<th>W17F*</th>
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<td>Kabsch-Sander</td>
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<td>Levitt-Greer</td>
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<td>27</td>
<td>56</td>
<td>26</td>
<td>38</td>
<td>24</td>
</tr>
</tbody>
</table>

WT, wild-type.

* Data adapted, with permission, from Gasymov OK, Abduragimov AR, Yusifov TN, Glasgow BJ. Binding studies of tear lipocalin: the role of the conserved tryptophan in maintaining structure, stability, and ligand affinity. Biochim Biophys Acta. 1999;1453:307–320.
domain at http://www.rcsb.org]). Ragona et al. have suggested that Trp19 and Tyr102 participate in internal and external hydrophobic clusters or clusters of residues that may stabilize the core of β-lactoglobulin. Mutation I98C in TL affected the near-UV CD spectrum in the region of 290 nm, a spectral region affected only by tryptophan. Ile98 has dynamic tertiary side-chain interactions with residues of at least one other strand. It is quite plausible that in TL, Trp17 on the A strand and Ile98 on the G strand participate in one or more hydrophobic clusters with other residues that contribute rigidity to TL. Relaxation of structure at the tip of the cavity may permit and Ile98 on the G strand participate in one or more hydrophobic clusters with other residues that contribute rigidity to TL. Relaxation of structure at the tip of the cavity may permit

FIGURE 10. Denaturation of TL and mutant proteins in urea monitored by CD at a wavelength of 218 nm. Protein concentration was 1 mg/mL in each case. The midpoints of transition (urea concentration at 50% change in ellipticity) are shown in parentheses. Retinol was added to apo-TL in a 2:1 molar ratio.

Our previous studies show that binding affinities of TL for fatty acids level off rather than decrease after the cavity-limiting chain length of 18 carbons is exceeded. In TL, binding affinity is dependent on the alkyl chain length rather than an interaction of the carboxyl group with a residue at the calyx mouth. The data in the present study, in combination with those of others, reveal that the binding affinities of retinoids to TL are only slightly altered by the type of R group of the retinoid. It is likely that the interaction of the R group with an amino acid at the TL calyx mouth minimally impacts binding affinity. TL has a minimally greater binding affinity for retinol than it does for either retinal or retinoic acid. In contrast to TL, β-lactoglobulin has a greater binding affinity for retinoic acid than retinol. Furthermore, substitution of Lys69 in β-lactoglobulin results in slightly lower affinity for retinoic acid but no change in the affinity for retinol. In the case of RBP the closest contacts to the retinol alcohol group are L35 and L97. There are no charged amino acids for an interaction with the functional group of retinoids that neutralize the ligand carboxylate. Arg80 is considered critical for ligand recognition. The double mutant E63/R80I shows an insignificant change of the Kd for retinoic acid compared with the wild type. However, unlike the wild type, E63I/R80I binds retinol and retinal. Hence, the amino acid composition at the mouth appears to determine ligand specificity, but ligand affinity is determined by the shape and hydrophobicity of the cavity. In

acids at the calyx mouth and the functional group of ligands may influence ligand affinity in lipocalins. In crystallographic studies, loose binding has been described between Lys69 of β-lactoglobulin and the carboxyl groups of 12-bromododecanoic and palmitic acids. However, in nuclear magnetic resonance (NMR) studies of β-lactoglobulin in solution, no charged interaction was observed between palmitic acid and residues at the mouth of the protein calyx.

Our previous studies show that binding affinities of TL for fatty acids level off rather than decrease after the cavity-limiting chain length of 18 carbons is exceeded. In TL, binding affinity is dependent on the alkyl chain length rather than an interaction of the carboxyl group with a residue at the calyx mouth. The data in the present study, in combination with those of others, reveal that the binding affinities of retinoids to TL are only slightly altered by the type of R group of the retinoid. It is likely that the interaction of the R group with an amino acid at the TL calyx mouth minimally impacts binding affinity. TL has a minimally greater binding affinity for retinol than it does for either retinal or retinoic acid. In contrast to TL, β-lactoglobulin has a greater binding affinity for retinoic acid than retinol. Furthermore, substitution of Lys69 in β-lactoglobulin results in slightly lower affinity for retinoic acid but no change in the affinity for retinol. In the case of RBP the closest contacts to the retinol alcohol group are L35 and L97. There are no charged amino acids for an interaction with the functional group of retinoids. In another example, epididyml retinoic acid–binding protein has a web of charged amino acids at the mouth of the cavity that neutralize the ligand carboxylate. Arg80 is considered critical for ligand recognition. The double mutant E63/R80I shows an insignificant change of the Kd for retinoic acid compared with the wild type. However, unlike the wild type, E63I/R80I binds retinol and retinal. Hence, the amino acid composition at the mouth appears to determine ligand specificity, but ligand affinity is determined by the shape and hydrophobicity of the cavity. In
these examples, the interactions of retinoids with lipocalins exemplify how variation in the interactions of the R group of ligands with amino acids at critical positions alter binding properties and confer functional diversity in the family.

Some investigators have suggested that because TL binds retinoids, it functions as a retinol carrier or as a scavenger for oxidation products. However, small amounts of RBP have been identified recently in human tears by mass spectroscopy and the message transcript for RBP has been identified recently in a human lacrimal gland cDNA library (Glasgow BJ, Yusifov TN, Abduragimov AR, et al., unpublished data, 2002). Retinol is bound to lacrimal-secreted RBP in rabbit tears. The binding affinities of TL obtained in this study for retinoids are very similar to those published for RBP. TL is present in normal tears, but it has not been detected bound to TL, although the concentrations may have been below the limit of detection for the analytic procedure. Rather, lipids with alkyl chains longer than that of retinol were discovered in abundance bound to TL from human tears. Because palmitic acid displaces retinoids from TL (Fig. 3), it is unlikely that TL could act as the principal carrier of retinol in tears.

Mutations at the tip of the cavity that produce structural relaxation in TL result in different responses to retinol versus fatty acid binding: Fatty acid binding is reduced but retinol binding is increased. The fundamental differences in response to relaxation of structure between these ligands may have functional implications for the role of TL in delivering ligands by mechanisms that involve structural changes.

Similarly, global structural relaxation induced by exposure to an acidic environment suggests mechanistic differences in retinol release from TL compared with RBP. Retinol is released from RBP at low pH by a mechanism that induces a molten globule state. Similarly, fatty acids are released from TL at low pH. A localized acidic microenvironment is produced by proton gradients created at lipid interfaces, such as are encountered in the tear film. However, the data in the current study show that the binding of retinol to TL did not change significantly at low pH and therefore TL could not deliver retinol by this mechanism. These findings suggest that in the absence of another specific releasing mechanism, TL would be more suitable to deliver fatty acids than retinol to the tear film interfaces.

In summary, the data show the influence of structural changes on ligand binding, specificity, and delivery of TL. For each lipocalin and each ligand, differences in function are likely to be dictated by the variation of amino acid residues at key positions, the interactions with functional groups, and the individual structural responses of each protein to different environments.

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