

Native Disulfide Bonds in Plasma Retinol-Binding Protein Are Not Essential for All-*trans*-Retinol-Binding Activity

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A human plasma retinol-binding protein (RBP) mutant, named RBP-S, has been designed and produced in which the six native cysteine residues, involved in the formation of three disulfide bonds, have been replaced with serine. A hexa-histidine tag was also added to the C-terminus of RBP for ease of purification. The removal of the disulfide bonds led to a decrease in the affinity of RBP for all *trans*-retinol. Data indicates all-*trans*-retinol binds RBP and RBP-S with $K_d = 4 \times 10^{-8}$ M and 1×10^{-7} M, respectively, at approximately 20 °C. RBP-S has reduced stability as compared to natural RBP below pH 8.0 and at room temperature. Circular dichroism in the far-UV shows that there is a relaxation of the RBP structure upon the removal of its disulfide bonds. Circular dichroism in the near-UV shows that in the absence of the disulfide bonds, the optical activity of RBP is higher in the 310–330 nm than in the 280–290 nm range. This work suggests that the three native disulfide bonds aid in the folding of RBP but are not essential to produce a soluble, active protein.

Keywords: human plasma retinol-binding protein • all-*trans*-retinol • genetic engineering • disulfide bonds

1. Introduction

Retinol-binding protein (RBP), a 21 kDa monomeric protein, is the principal carrier of all-*trans*-retinol (vitamin A). RBP is synthesized in the liver and is secreted into the blood stream upon binding of all-*trans*-retinol.¹ Analysis of the structure of RBP shows that it is a member of the lipocalins, a superfamily that has been used extensively as a marker of diseases associated with inflammation, cancer, lipid disorders, and kidney and liver functions.² For example, plasma levels of the lipocalins α -1-acid glycoprotein and glycodelin are associated with acute phase response and show immunomodulatory and antiinflammatory properties.³ Variations in concentration and modification of apolipoprotein D are correlated with its expression in distinct cancer forms⁴ as well as in lipid disorders.⁵ RBP and α -1-microglobulin are widely used for the diagnosis of kidney disease. RBP is also utilized as a marker for hepatocytic growth and regeneration. This illustrates the usefulness of lipocalins in preventive medicine and suggests the importance of understanding their properties and structural characteristics.

In this work, we characterize the stability and all-*trans*-retinol binding properties of RBP by altering residues critical to its secondary structure. The known three-dimensional structure of RBP^{6,7} indicates that this protein has an antiparallel β -barrel structure with a highly hydrophobic pocket, where RBP binds all-*trans*-retinol with a $K_d = 1.9 \times 10^{-7}$ M at 27 °C.⁸ RBP, unlike most lipocalins that are presumed to have either one or two

disulfide bonds,⁹ has six cysteine residues that are involved in the formation of three disulfide bonds.¹⁰ Analysis of its crystallographic structure reveals that the disulfide bonds formed by C4–C160 and C70–C174 are almost totally exposed to the solvent, whereas the remaining one, C120–C129, is slightly buried within the protein. The disulfide bond C4–C160, which covalently links the N- and C-terminus regions of RBP, is a feature present in nearly all lipocalins⁹ that makes the structure of RBP compact by bringing the α -helix closer to one of its β -sheets. The disulfide bond C70–C174 connects the end of one beta sheet with a flexible region of RBP to provide the structure with more rigidity. The disulfide bond C120–C129, which connects two neighboring strands near a loop, is the closest to the core of the β -barrel region. This loop is at the open end of the β -barrel, pointing away from the ligand-binding pocket, and has no direct contact with all-*trans*-retinol. Since there is no apparent interaction between the disulfide bonds of RBP and all-*trans*-retinol, it would be of interest to investigate if RBP is able to fold and preserve the affinity for its natural ligand in the absence of its disulfide bonds. Therefore, we eliminated the disulfide bonds in RBP by substituting its six cysteine residues with serine and characterized the properties of this construct.

2. Materials and Methods

Construction of Expression Vectors. Two expression vectors were constructed by using pCDL-SR α 296 (a kind gift from Dr. J. Ø. Moskaug) carrying a cloned RBP gene¹¹ as a starting material. An initial construct, carrying an unmodified RBP gene with a hexa-histidine tag, fused to the codon for Leu183 (AAC),¹² was cloned into pET-3a. For this construct, the DNA sequence

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(CAT-CAT-CAT-CAC-CAT-CAC-TAG) encoding a hexa-histidine tag with a translation termination codon ((His)₆-Stop), was used. In the other construct, a PCR (polymerase chain reaction)-based site-directed mutagenesis method was used on RBP to replace the six cysteine residues (C4 (TGC), C70 (TGC), C120 (TGC), C129 (TGT), C160 (TGC), C174 (TGC)) with serine (AGC). Both coding sequences were cloned separately between the *Nde* I and *Bam*H I sites of the plasmid pET-3a under the control of the ϕ 10 promoter of bacteriophage T7.¹³ The resulting expression vectors pRBP and pRBP-S encode, respectively, RBP, which consists of residues 1–183 of retinol-binding protein¹² with an added (His)₆ sequence at its C-terminus, and RBP-S, which is similar to RBP, but in which all cysteine residues have been replaced with serine.

Expression and Purification of RBP and RBP-S. Expression of RBP and RBP-S was carried out as previously described¹⁴ by using the *Escherichia coli* strain BL21(DE3)(pLysE)¹³ carrying pRBP and pRBP-S, respectively. Insoluble fractions were prepared from cell lysates and solubilized in 6 M guanidine-HCl pH 1.5. Samples were resuspended in a volume equal to the culture volume and loaded into dialysis tubing with a molecular weight cutoff of 10 kDa. Solubilized samples were dialyzed separately against one of four different buffers: 100 mM ammonium acetate-HCl pH 6.0, 100 mM Tris-HCl pH 7.5, 100 mM Tris-HCl pH 8.0, and 100 mM diethanolamine-HCl pH 9.3, to renature the proteins. Crude RBP and RBP-S solutions, prepared above, containing 0.5 M NaCl, 2 mM imidazole, 20 mM Tris-HCl pH 8.0, were applied separately to a nickel-chelating agarose column, made up from swell-gel nickel chelating disks (Pierce), equilibrated with the same buffer. After unbound materials were removed by washing the column with 0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl pH 8.0, bound proteins were eluted with 150 mM NaCl, 20 mM EDTA, 20 mM Tris-HCl pH 8.0. Eluted proteins were dialyzed against 10 mM NaCl, 1 mM Na-phosphate buffer pH 8.0 or 150 mM NaCl, 20 mM Na-phosphate buffer pH 8.0 and stored at 4 °C.

Mass Spectrometry. Samples of purified RBP and RBP-S were analyzed by matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) using a Shimadzu/Kratos Axima CFR in the linear mode. The best results were obtained with a matrix of α -cyano-4-hydroxycinnamic acid and a protein concentration of roughly 10 pmol/ μ L. Lysozyme was used as an external calibrant. All masses were averages determined for smoothed data (20 point, Savitsky-Golay) for duplicate acquisitions.

Immobilization of All-*trans*-Retinol on Cysteine-Agarose. Approximately 8 mg (36.9 μ mol) of PMPI (N-(p-maleimido-phenyl)isocyanate) (Pierce) dissolved in 100 μ L of dimethyl sulfoxide (DMSO) was combined with 1.06 mg (3.69 μ mol) of all-*trans*-retinol. The reaction mixture was incubated at room temperature (22 °C) for 3 h with shaking in the dark to allow the isocyanate group of PMPI to react with the hydroxyl group of all-*trans*-retinol. The resulting mixture was then added to approximately 0.3 μ mol of cysteine-agarose (Sigma), which was pretreated with 1 mM TCEP-HCl (tris(2-carboxyethyl)phosphine hydrochloride), in 0.9 mL of 0.6 M sodium phosphate pH 7.2 to ensure that the sulfhydryl group of cysteine reacts with the maleimide moiety of PMPI. The immobilization reaction was carried out with shaking at room temperature overnight. The reaction mixture was then loaded onto a column and washed extensively with 100% DMSO followed by 100% ethanol. The final product, all-*trans*-retinol-cysteine-agarose, was stored in 150 mM NaCl, 20 mM Tris-HCl pH 8.0 at 4 °C.

Determination of All-*trans*-Retinol-Binding Activity of RBP and RBP-S. Approximately, 50 μ g and 25 μ g of RBP and RBP-S eluted from nickel-chelating agarose, respectively, were applied separately to an all-*trans*-retinol-cysteine-agarose column equilibrated with 500 mM NaCl and 20 mM Tris-HCl, pH 8.0. After unbound materials were removed using the equilibration buffer, bound proteins were eluted from all-*trans*-retinol-cysteine-agarose by unpacking the column and incubating the mixture with SDS loading buffer (3% sodium dodecyl sulfate, 40% glycerol, 100 mM Tris-HCl pH 6.8). The samples were boiled for 5 min and analyzed by SDS-PAGE.¹⁵ To measure the nonspecific binding properties of cysteine-agarose, we applied an equal amount of each protein to a cysteine-agarose column and washed as described above. Then, the samples were boiled for 5 min and analyzed by SDS-PAGE.

Determination of the Dissociation Constant between All-*trans*-Retinol and RBP and RBP-S. Fluorescence binding assays were performed essentially following published methods^{8,14} with small variations. Data were obtained using a Hitachi F-2500 fluorescence spectrophotometer using a 1-cm filter of 2 M NaNO₂. The excitation wavelength was set at 334 nm. Separate containers carrying 32 mL of 1 μ M purified RBP and RBP-S in 150 mM NaCl, 20 mM sodium phosphate pH 8.0 were titrated with 5–10 μ L aliquots of 0.5 μ M of all-*trans*-retinol dissolved in 100% ethanol. Following each all-*trans*-retinol addition, 1.2 mL-aliquots were transferred to microcentrifuge tubes stored on ice to ensure the stability of the proteins, in particular RBP-S. After all samples were prepared, each sample was brought to room temperature (~20 °C) and the fluorescence was determined. To account for the natural fluorescence of all-*trans*-retinol, a solution containing *N*-acetyl-L-tryptophanamide having an absorbance at 296 nm equal to the protein concentration at 280 nm was used.⁸ *N*-acetyl-L-tryptophanamide possesses a typical protein fluorescence and does not interact with all-*trans*-retinol. The dissociation constants between the *trans*-retinol-protein complexes were determined by a linear least-squares plot.⁸

Circular Dichroic Spectral Measurements. Spectra were recorded (Aviv 62DS spectrometer, 1 mm path length for far-UV spectra and 10 mm path length for near-UV spectra) using RBP and RBP-S concentrations of 6 μ M and all-*trans*-retinol concentrations of 15 μ M with RBP and 40 μ M with RBP-S. Data were averaged over 5 s and collected using 1 nm intervals. Data were obtained at 4 °C. CD spectra of RBP and RBP-S were corrected by subtracting the solvent spectrum obtained under identical conditions. CD spectra of all-*trans*-retinol-RBP and all-*trans*-retinol-RBP-S complexes were corrected by subtracting the all-*trans*-retinol-solvent spectrum obtained under identical conditions. Far-UV and near-UV measurements were obtained in the 196–250 and 250–420 nm range, respectively. Computer-assisted estimation of α -helix and β -sheet content of RBP, RBP-S, all-*trans*-retinol-RBP, and all-*trans*-retinol-RBP-S complexes were obtained using a neural network algorithm.¹⁶

3. Results and Discussion

There are four different functions associated with the formation of disulfide bonds in proteins: folding (e.g., bovine pancreatic trypsin inhibitor), enhancement of stability (e.g., β -lactamase), catalytic activity, which leads to the formation and cleavage of a disulfide bond (e.g., ribonucleotide reductase), and promoting a folding pathway (e.g., P22 tailspike).¹⁷ Although there are numerous studies in these four areas, there

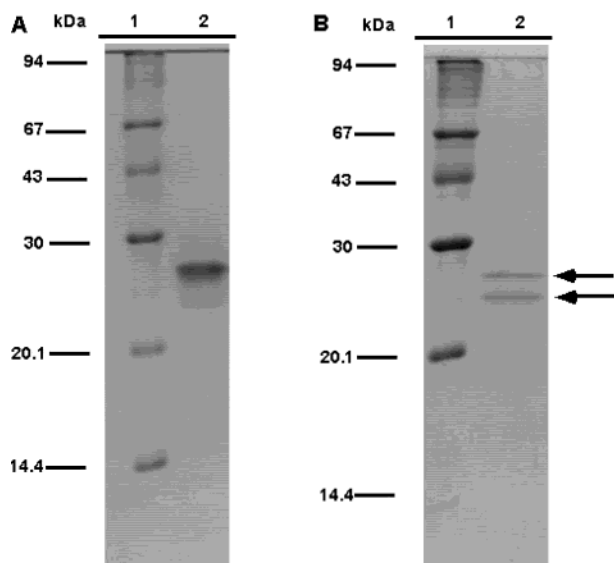


Figure 1. SDS-PAGE analysis of RBP and RBP-S. (A) Lane 1: molecular mass standard proteins; Lane 2: RBP. Sample in lane 2 was preincubated for 30 min with 20 mM 2-mercaptoethanol prior to loading. (B) Lane 1: molecular mass standard proteins; Lane 2: RBP-S. Unproteolyzed RBP-S (top) and proteolyzed RBP-S (bottom) are shown by arrows.

is particular interest in understanding the contribution of disulfide bonds to protein stability. Most efforts to modulate stability employ one of two strategies: the introduction of non-native cysteine residues to make disulfide bonds,^{18,19} and the elimination of disulfide-bonded cysteine residues.^{19,20} In this work, we used the second approach.

Analysis of the three-dimensional structure of RBP^{6,7} suggested that the disulfide bonds are not involved in the binding or contact with the natural ligand of RBP, all-*trans*-retinol. The disulfide bonds appeared to enhance the stability of this protein by topologically constraining the residues forming the β -barrel restricting the exposure of the buried hydrophobic pocket to the solvent. Molecular simulation analysis with the protein modeling program QUANTA (Molecular Simulations, Inc.; CA), using the known three-dimensional structure of RBP, predicted that the replacement of the six cysteine residues with serine would not introduce any steric effects and likely maintain the properties of RBP. Accordingly, we genetically engineered two constructs containing the RBP gene.

Construction, Expression, Purification, and Confirmation of the Molecular Weight of RBP and RBP-S. We constructed an expression vector for retinol-binding protein, RBP, which consists of residues E1 to L183 of retinol-binding protein¹² and has an additional hexa-histidine tag fused at its C-terminus. A second construct, RBP-S, is identical to RBP except that all cysteine residues were replaced with serine. Both constructs were expressed efficiently in *E. coli* by using the T7 expression system. SDS-PAGE analysis of an equal amount of cells revealed that a similar amount of protein was produced by both strains (data not shown).¹³ RBP and RBP-S were purified to homogeneity by a procedure that includes nickel-chelate affinity chromatography (Figure 1). Genes were verified by sequencing and analyzing the expressed products by MALDI-TOF-MS. The mass of full length RBP was 21 989 Da by singly charged molecular ion and 22 016 Da by doubly charged, giving an error of -30 and -3 Da, respectively, relative to the theoretical value. Corresponding results for the full length RBP-S were 21 913 and

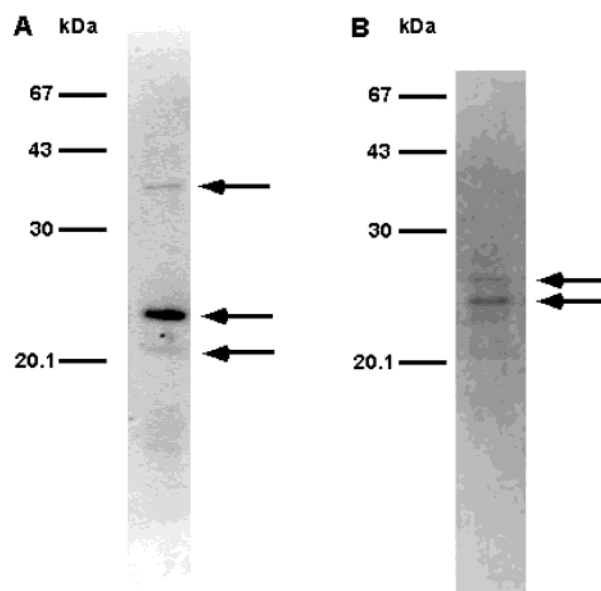


Figure 2. SDS-PAGE analysis of RBP and RBP-S bound to all-*trans*-retinol-cysteine-agarose. (A) Dimerized RBP (top), full-length RBP (middle) and proteolyzed RBP (bottom) are shown by arrows. (B) Full-length RBP-S (top), and proteolyzed RBP-S (bottom) are shown by arrows.

21 924 Da, or -16 and -5 Da, respectively, relative to theoretical. The average difference between RBP and RBP-S of 84 Da corresponds to a replacement of 5.6 cysteine residues with serine. Given the accuracy of these determinations, the results are entirely consistent with fully modified RBP-S.

Ligand Binding of RBP and RBP-S. To verify that the purified proteins were active, aliquots of both proteins were incubated with all-*trans*-retinol that was immobilized *via* the heterobifunctional cross-linker PMPI on cysteine-agarose. Bound proteins were analyzed by SDS-PAGE (Figure 2). As a control, we tested the nonspecific binding properties of RBP and RBP-S to cysteine-agarose. Our results indicate that approximately 10% of the total bound protein is nonspecifically bound to the cysteine-agarose, which suggests that RBP and RBP-S were specifically captured by all-*trans*-retinol immobilized on cysteine-agarose. Elution of RBP from all-*trans*-retinol-cysteine-agarose revealed the presence of three bands (Figure 2A). The strongest band (middle band) corresponded to the full-length RBP. The two other bands, of lower intensity, corresponded to a proteolyzed monomer (bottom band) and to an RBP dimer (top band), that likely results from intermolecular disulfide bond formation between misfolded RBP molecules. This last result is significant because it shows that even in the absence of proper disulfide bond formation, RBP is still able to bind all-*trans*-retinol. A small amount of dimer was also seen by MALDI-TOF-MS, although the process creating it could not be identified; no significant level of truncated monomer was detected. In the case of RBP-S (Figure 2B), only two bands were observed. One band corresponded to the full-length RBP-S and another to a proteolyzed product of this protein. Because both proteolyzed products were isolated by nickel-chelate affinity chromatography using a histidine tag fused at their C-terminus, these results suggest that the purified proteins were proteolyzed at their amino end. The ability of these reduced-size proteins to bind all-*trans*-retinol can be explained by analysis of the three-dimensional structure of natural RBP, which shows that the first 21 amino acids at the N-terminus are not part of the

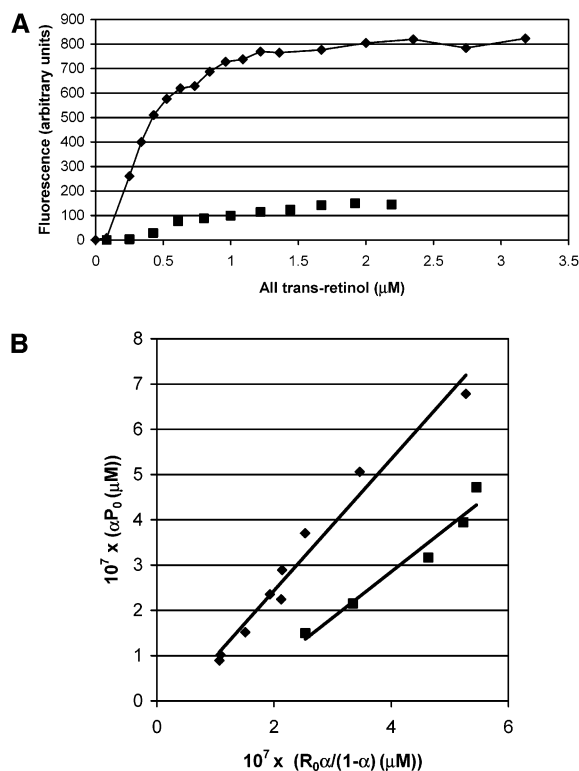


Figure 3. Binding of all-*trans*-retinol to RBP-S followed by the increase in fluorescence intensity. Excitation wavelength was set to 334 nm. Emission observed through a 1-cm layer of 2 M NaNO₂. The titration consisted of 1.2 mL of 1.0 μM of RBP-S in 150 mM NaCl, 20 mM sodium phosphate pH 8.0 with various amount of all-*trans*-retinol. (A) Observed fluorescence intensity of all-*trans*-retinol-RBP complex (◆); observed fluorescence intensity of all-*trans*-retinol-RBP-S complex (■). (B) Linear least-squares plots for the determination of the association constant of RBP (◆) and RBP-S (■) with all-*trans*-retinol. Free binding sites, αP₀, where “α” is the fraction of free binding sites and “P₀” is the total protein concentration, are plotted versus R₀α/(1 - α), where R₀ is the total all-*trans*-retinol concentration.

core β-barrel structure and not involved in ligand binding. Therefore, our results indicate that RBP is able to bind all-*trans*-retinol in the absence of disulfide bonds. These results are in agreement with preliminary data that indicates that the substitution of cysteine residues of RBP yields an active protein.²¹

RBP and RBP-S were not soluble and contained within inclusion bodies. SDS-PAGE analysis of an equal amount of cells revealed that a similar amount of protein was produced by both strains (data not shown). Upon renaturation, the yields of RBP and RBP-S were approximately 1.2 mg and 0.1 mg per 100 mL of culture, respectively. Although the removal of the disulfide bonds reduced the protein yield significantly, it was possible to obtain a soluble, active RBP mutant by renaturing this protein against 100 mM diethanolamine-HCl pH 9.3. To improve the yield of RBP-S, we attempted the renaturation step under different pH conditions (pH 6, 7.5, 8). Despite this optimization procedure, best results were still obtained at pH 9.3, using the same conditions used for unmodified RBP.¹⁰

We observed that RBP-S had reduced stability below pH 8.0, and at room temperature, as judged by a slow increase in turbidity when the protein was brought from 4 °C to room temperature. However, this protein was remarkably stable at

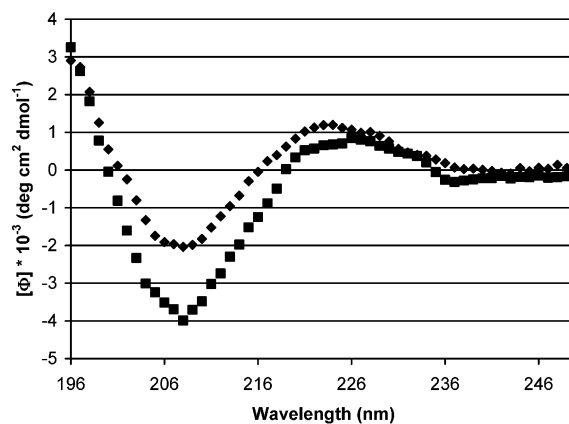


Figure 4. Far-UV CD spectra of apo-RBP and all-*trans*-retinol-RBP complex. Apo-RBP (◆) and all-*trans*-retinol-RBP complex (■) (6 μM) were prepared in 10 mM sodium chloride, 1 mM sodium phosphate buffer, pH 8.0.

Table 1. Predicted Secondary Structure

	α-helix (%)	antiparallel β-sheet (%)	parallel β-sheet (%)	β-turn (%)	random coil (%)
RBP	10	16	15	17	42
RBP + all- <i>trans</i> -retinol	10	16	15	17	42
RBP-S	10	16	15	17	42
RBP-S + all- <i>trans</i> -retinol	12	15	14	18	41

pH 8.0 and remained active at room temperature, after storage at 4 °C for over three weeks.

Dissociation Constants. To ensure the stability of RBP-S while measuring the fluorescence of the all-*trans*-retinol-RBP-S complex, data was collected using several samples containing 1 μM of RBP and RBP-S, separately, and various amounts of all-*trans*-retinol. We observed that during the data collection, the solution containing RBP-O remained clear with no detectable turbidity. However, when the concentration of all-*trans*-retinol exceeded approximately 2.5 μM, a precipitate was detected in the RBP-S solution. By using the fluorescence intensity measurements of the complexes, corrected for the natural fluorescence of all-*trans*-retinol (Figure 3), we determined, by a least-squares plot, the dissociation constant between all-*trans*-retinol and RBP and RBP-S. Our measurements indicated a K_d = 4 × 10⁻⁸ M and 1 × 10⁻⁷ M for RBP and RBP-S, respectively. These results suggest that the disulfide bonds of RBP confer stability to this protein but are not essential for all-*trans*-retinol-binding activity. The value obtained for RBP is approximately five times larger than that reported.⁴ This difference is likely due to the different temperature conditions under which the experiments were carried out.

Circular Dichroism. The far-UV spectra provided information about the secondary structure of RBP and RBP-S and the protein complexes formed with all-*trans*-retinol. The CD spectra of RBP and all-*trans*-retinol-RBP indicated minima at 208 nm and crossover at about 200 and 220 nm indicative of a β-sheet structure (Figure 4). The data showed that the complex preserved the β-structure of RBP and had a higher signal than RBP. Computer analysis agreed with the circular dichroism data (Table 1) and showed that the addition of all-*trans*-retinol binding had no effect on the secondary structure of RBP. The CD spectra of RBP-S and all-*trans*-retinol-RBP-S maintained the minimum near 208 nm and crossover at about 200 nm;

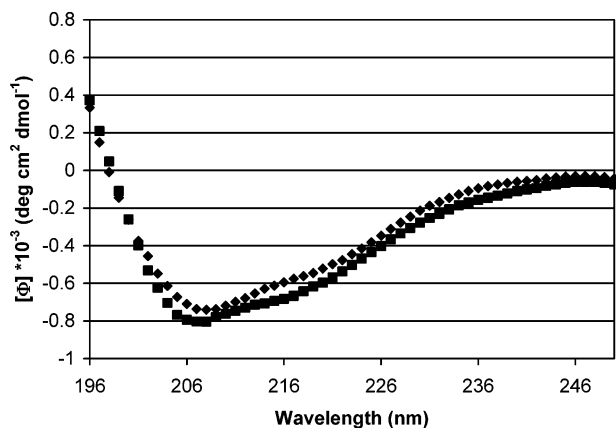


Figure 5. Far-UV CD spectra of apo-RBP-S and all-*trans*-retinol-RBP-S complex. Apo-RBP-S (◆) and all-*trans*-retinol-RBP-S complex (■) (6 μ M each sample) were prepared in 10 mM sodium chloride, 1 mM sodium phosphate buffer, pH 8.0.

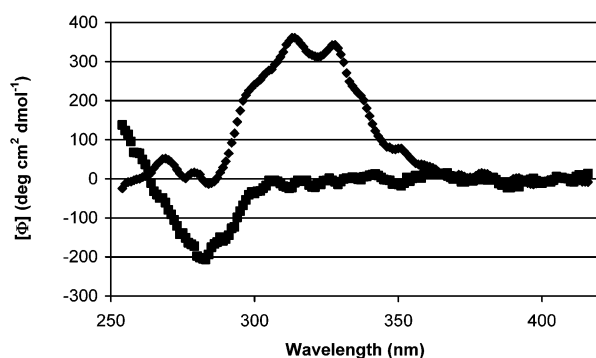


Figure 6. Near-UV CD spectra of apo-RBP and all-*trans*-retinol-RBP complex. Apo-RBP (◆) and all-*trans*-retinol-RBP complex (■) (6 μ M each sample) were prepared in 10 mM sodium chloride, 1 mM sodium phosphate buffer, pH 8.0.

however, the data clearly showed that the CD spectra were attenuated (Figure 5) and there was no crossover at 220 nm. The data also showed the spectral minimum of RBP-S was broader. The all-*trans*-retinol-RBP-S complex produced a higher signal than RBP-S. Computer analysis showed that RBP-S and RBP have similar β -sheet structures. Data also indicated that part of the β -structure was slightly affected upon binding of all-*trans*-retinol (Table 1). This suggested that the removal of the disulfide bonds relaxed the structure that RBP-S adopts when it binds all-*trans*-retinol.

The near-UV spectra of RBP (Figure 6) showed two maxima near 313 and 328 nm. These peaks also appear in the near-UV spectrum of RBP-S (Figure 7) but they had approximately five times lower intensity. The data also shows that there are two additional peaks near 280 and 290 nm in the RBP-S spectrum. The reduced optical activity of RBP-S suggests that the removal of the disulfide bonds relaxed the structure of RBP and led to a decrease in binding to all-*trans*-retinol. Analysis of the near-UV differential spectra of RBP and RBP-S complexed with all-*trans*-retinol indicates the degree of optical activity in the 280–290 nm region, indicative of conformational changes in aromatic residues, was not as pronounced as that observed in the 310–330 nm spectra (Figure 8). This result differs from data reported on a tear lipocalin,⁹ where the greatest alteration of the spectra upon all-*trans*-retinol binding occurs in the 280–290 nm region.

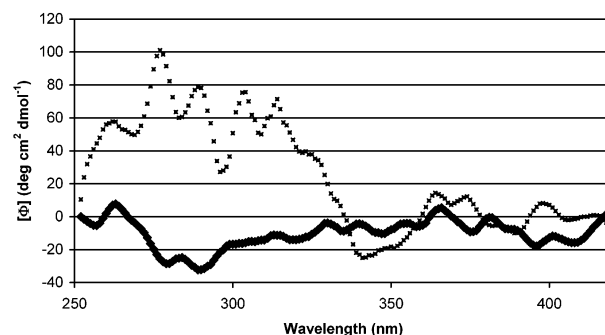


Figure 7. Near-UV CD spectra of apo-RBP-S and all-*trans*-retinol-RBP-S complex. Apo-RBP-S (◆) and all-*trans*-retinol-RBP-S complex (■) (6 μ M each sample) were prepared in 10 mM sodium chloride, 1 mM sodium phosphate buffer, pH 8.0.

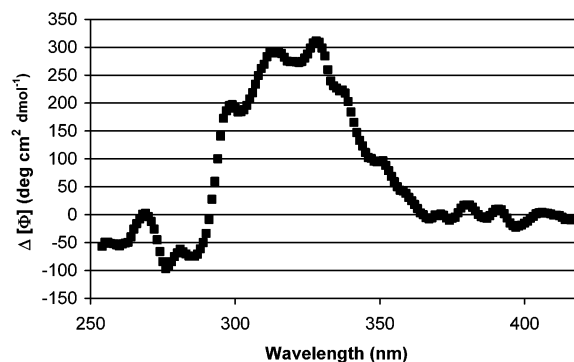


Figure 8. Differential Near-UV CD spectra of *trans*-retinol-RBP and all-*trans*-retinol-RBP-S complexes. Data were obtained by subtracting the spectra of all-*trans*-retinol-RBP-S from all-*trans*-retinol-RBP (■) complexes shown in Figures 6–7.

4. Conclusions

The addition of a hexa-histidine tag at the C-terminus of RBP provided a simple method for the purification of this protein. The substitution of the six cysteine residues with serine produced a RBP mutant that is capable of folding in the absence of the three disulfide bonds that are present in the native protein. Experimental data show that the removal of the disulfide bonds reduces the affinity for all-*trans*-retinol-binding, leading to an approximate two and a half higher dissociation constant. Our observations also show that the removal of the disulfide bonds adversely affects the stability of the protein at room temperature; however, RBP-S remains remarkably stable and active at pH 8.0 after storage at 4 $^{\circ}$ C for over three weeks. Circular dichroism data indicates that the removal of the disulfide bonds leads to a relaxation of the RBP structure. This effect is most prominent in the 310–330 nm region of the spectra. These results suggest that the disulfide bonds of RBP aid in folding and confer stability to RBP, but are not essential for producing a soluble RBP that binds all-*trans*-retinol.

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