Isolation and Characterization of Monkey Interphotoreceptor Retinoid-Binding Protein, a Unique Extracellular Matrix Component of the Retina

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ABSTRACT: The interphotoreceptor retinoid-binding protein (IRBP) has been isolated from monkey interphotoreceptor matrix (IPM). Following gentle washing of the IPM from the retinal surface, the protein was purified to homogeneity by concanavalin A–Sepharose affinity chromatography, ion-exchange high-performance liquid chromatography (HPLC), and size-exclusion HPLC. Bovine IRBP was purified similarly and compared with the monkey protein. Sedimentation equilibrium analysis yielded a molecular weight of 106 000 ± 2900 for the native monkey protein. Sedimentation velocity analysis gave a sedimentation coefficient of 5.4 ± 0.3 S and a frictional ratio of 1.59, indicating an asymmetrical molecular shape. IRBP contains neutral sugar, including fucose, and sialic acid; the glycoprotein nature of the proteins probably accounts for the microheterogeneity observed in the electrofocusing pattern of both bovine and monkey IRBP. Both IRBPs have isoelectric points between 6.0 and 7.0. The fluorescence emission λ_{max} of the bound ligand was 470 nm with excitation at 340 nm, while the excitation λ_{max} was 333 nm with emission at 470 nm, for monkey IRBP incubated with exogenous *all-trans*-retinol. The amino acid compositions of the monkey and bovine proteins are similar; nonpolar amino acids account for over 50% of the residues, which may explain the apparent hydrophobic nature of the isolated proteins. The amino-terminal analyses indicated considerable homology between the monkey and bovine IRBPs in this region and verified the purity of the isolated proteins. IRBP thus appears to be a unique, conserved glycoprotein of the retinal extracellular matrix that could serve as a retinoid-transport vehicle.

Intercellular and intracellular transport of vitamin A in the retina–pigment epithelium complex of the eye is thought to be mediated by a number of retinoid-binding proteins [for a review, see Chader (1982)]. Vitamin A is stored in the pigment epithelium (PE)[1] and must be transported to the retinal photoreceptor cell for use in the visual cycle (Dowling, 1960). Following bleaching of rhodopsin, the released retinoid must find its way from the photoreceptor outer segment back to the pigment epithelium. Most of this intercellular transport probably occurs through the interphotoreceptor space (IPS; Feeney, 1973), perhaps via a unique interphotoreceptor matrix glycoprotein termed interphotoreceptor retinoid-binding protein (IRBP; Lai et al., 1982; Bunt-Milam & Saari, 1983). IRBP is associated with the retina–PE complex of several species (Wiggert et al., 1976). Some of its characteristics have already been described in frog (Wiggert et al., 1979), rabbit (Lai et al., 1982), and cow (Wiggert et al., 1977; Adler & Klucznik, 1982; Adler & Martin, 1982; Liou et al., 1982; Chader et al., 1983; Bridges et al., 1983; Adler & Evans, 1983; Fong et al., 1984). In immunocytochemical studies, Bunt-Milam & Saari (1983) have shown that IRBP is localized to the interphotoreceptor matrix between the PE cell layer and the retinal photoreceptor and Müller (glial) cells.

Recently, Pfeffer et al. (1983) have shown this glycoprotein to be the major soluble protein and the only retinoid-binding protein present in the monkey interphotoreceptor matrix. Furthermore, Wiggert et al. (1984) have shown that the protein is synthesized by monkey retina in culture and secreted into the culture medium and that such secretion is blocked by monensin, an ionophore known to inhibit glycoprotein secretion at the level of the golgi complex (Tartakoff & Vassalli, 1977). This protein is thus of interest not only in regard to the visual process but also as a readily accessible, rapidly synthesized, soluble extracellular matrix component.

In the present paper, we (1) describe a rapid procedure for the purification of monkey IRBP, (2) characterize several of the important features of the purified protein, and (3) compare some of the properties of the monkey IRBP with those of bovine IRBP purified in an identical manner.

Experimental Procedures

Interphotoreceptor Matrix (IPM) Wash. Rhesus monkey (*Macacca mulatta*) eyes were obtained from animals (male and female, 1–3 years) killed by exsanguination while under deep barbiturate anesthesia. The eyes were rinsed briefly in Dulbecco's phosphate-buffered saline, pH 7.4 (Gibco), the anterior segment was dissected away, and as much vitreous as possible was removed. The retinas were dissected from the underlying pigment epithelium and, individually, gently swirled in Dulbecco's phosphate-buffered saline, 0.5 mL per retina, to remove soluble interphotoreceptor matrix components. All procedures were carried out at 4 °C. The wash was then ultracentrifuged at 110000*g* for 30 min. The supernatant,

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[1] Abbreviations: Con A–Sepharose, concanavalin A–Sepharose; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography, IPM, interphotoreceptor matrix; IRBP, interphotoreceptor retinoid-binding protein; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; PE, pigment epithelium.

containing the crude IRBP, was used immediately or stored frozen at −70 °C until required. Early isolation experiments showed no difference between results in the presence or absence of 1 mM phenylmethanesulfonyl fluoride (PMSF), a protease inhibitor. Because of its lack of effect, we did not use PMSF in the experiments reported here. For comparative purposes, bovine eyes were obtained fresh from a local slaughterhouse and the IPM wash was prepared in the same manner as described above. Alternatively, frozen bovine retinas (Hormel, Inc.) were used. In this case 50 frozen retinas were thawed quickly in phosphate-buffered saline in a 23 °C water bath with gentle swirling as described above.

Affinity Chromatography. The crude IPM wash containing the IRBP (Pfeffer et al., 1983) was subjected to concanavalin A–Sepharose (Con A) affinity chromatography in a batch-type column technique. A Pharmacia K9-15 column was half-filled with 4 mL of Con A–Sepharose gel (Pharmacia Fine Chemicals) and equilibrated with 10 volumes of the starting buffer, 50 mM Tris, pH 7.6, and 140 mM NaCl [after Adler & Klucznik (1982)]. Up to 30 mL of IPM wash was applied to the column and run through at a flow rate of about 0.5 mL/min. Unbound material was washed off with 5 volumes of starting buffer. Bound material was eluted with 1.5–2.0 volumes of 50 mM methyl α-D-mannoside in starting buffer. The elution buffer was run through until 1 mL was collected and the flow was stopped for 2 h. Elution was then resumed and the remaining eluate was collected. Protein concentration in the fractions was estimated by measurement at 280 nm.

High-Performance Liquid Chromatography (HPLC). Ion-exchange HPLC was carried out by using a Pharmacia FPLC system equipped with a Mono-Q anion-exchange column. Approximately half of the methyl α-D-mannoside eluate (starting with the 30-mL IPM wash) was applied directly to the column, and chromatography was carried out at ambient temperature by using 10 mM Tris buffer, pH 7.5, containing 2 mM EDTA and a 0–0.5 M NaCl gradient at a flow rate of 0.5 mL/min. Fractions (500 μL) were collected and elution was monitored at 280 nm. IRBP was identified by SDS–PAGE electrophoresis and [^{3}H]retinol binding. Size-exclusion chromatography was performed by using a Beckman-Altex Model 324 liquid chromatography system equipped with a Hitachi UV–visible spectrophotometer (Model 155-10/40), using two Spherogel TSK 3000 SW size-exclusion columns (7.5 × 300 mm) connected in series and protected by a Spherogel TSK 2000SW precolumn. The mobile phase consisted of 40 mM phosphate, pH 7.0, 160 mM Na_2SO_4, 1.6 mM EDTA, and 20% glycerol (v/v). The peak from the ion-exchange column was injected (sample volume up to 500 μL) and chromatographed at a flow rate of 0.3 mL/min at room temperature, 150-μL fractions were collected, and the elution profile was followed at 280 nm. The IRBP peak, identified by [^{3}H]retinol binding and SDS–PAGE (Pfeffer et al., 1983), was stored at −70 °C.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE was performed by using a Bethesda Research Laboratories vertical slab gel system (Pfeffer et al., 1983). Molecular weight standards were supplied by Bio-Rad Instruments (Rockville Center, NY). Some gels were silver-stained by using a Bio-Rad silver-staining kit.

Isoelectric Focusing. Analytical isoelectric focusing was carried out by using 6.2% acrylamide with 5% ampholyte (Pharmalyte pH 3–10; Pharmacia Fine Chemicals) in cylindrical rod gels. The cathode buffer consisted of 3 mL of triethanolamine in 750 mL of water, and the anode buffer was 1.5 mL of concentrated H_2SO_4 in 750 mL of water. Samples were dialyzed against 50 mM Tris, pH 7.6, mixed with 0.15 volume of pH 3–10 Pharmalyte, and focused for 16 h at 200 V at 10 °C. The pH gradient obtained was monitored by eluting sequential 5-mm slices of a blank gel in 1 mL of water. Gels were stained for protein by using a procedure that reduces nonspecific staining due to ampholytes (Righetti & Drysdale, 1974).

Fluorescence Studies. Samples were prepared by incubating the peak from ion-exchange HPLC with 8×10^{-7} M *all-trans*-retinol (Sigma Chemical Co., St. Louis, MO) overnight, followed by size-exclusion HPLC of the sample as described above. Fluorescence spectroscopy was carried out by using an Aminco SPF-500 spectrofluorometer in the ratio mode.

Carbohydrate Analysis. Neutral sugar content was determined by the method of Roe (1955). The monkey IRBP samples for assay were dialyzed exhaustively against three changes of water from a Milli-Q reagent water system (Continental Water Systems) and then lyophilized. The IRBP was then weighed and redissolved in 50 mM phosphate buffer, pH 7.0. Fucose was determined by the procedure of Dische & Shettles (1948). Hexose standards used were D-(+)-mannose, D-(+)-galactose, and D-(+)-fucose. Sialic acid content of the purified bovine IRBP was assayed by the method of Warren (1959) following liberation of the sialic acid by 0.1 N H_2SO_4. *N*-Acetylneuraminic acid from sheep submaxillary gland (Sigma Chemical Co.) was used as a standard.

Ultracentrifugational Analysis. The molecular weight of the IRBP was determined by sedimentation equilibrium analysis using a Beckman Model E analytical ultracentrifuge equipped with a multiplexed ultraviolet absorption scanner. Three samples of IRBP were run simultaneously for 90 h at 12000 rpm and 5 °C with initial concentrations giving optical densities at 280 nm of 0.057, 0.114, and 0.171, respectively. The buffer was 0.05 M sodium phosphate, pH 7.0, $\rho = 1.007$ g/cm^3 at 5.0 °C. The partial specific volume was calculated from the amino acid composition and the sugar composition. The sedimentation coefficient was determined by sedimentation velocity analysis in the same instrument. Three samples were run simultaneously at 40000 rpm and 20 °C. The scanner traces were digitized manually, and the ultracentrifuge data were analyzed by mathematical modeling using the MLAB system operating on a DEC-10 computer to do nonlinear least-squares curve fitting to appropriate models.

Preparation of IRBP for Structural Analysis. IRBP (about 0.5 mg in 1 mL) was precipitated by the addition of 1 mL of 20% trichloroacetic acid. The precipitate was washed and resuspended in 100 μL of glacial acetic acid and incubated at 37 °C for 30 min. H_2O (200 μL) was added to make a final concentration of 30% acetic acid in which both monkey and bovine IRBP are soluble.

Amino Acid Analysis. Protein was hydrolyzed by 6 N HCl at 115 °C for 22 h and analyzed by using a Beckman 121M amino acid analyzer. The quantities of methionine and half-cystine were determined by the amounts of methionine sulfone and cysteic acid formed by performic acid oxidation (Oliveira et al., 1979) prior to HCl hydrolysis. The values reported are the averages of at least three determinations. Values of serine and threonine are uncorrected.

Amino-Terminal Analysis. Automated Edman degradation was utilized with conversion of the unstable intermediates to stable phenylthiohydantoin amino acids (Edman & Begg, 1967) in an Applied Biosystems Model 470A gas-phase protein sequencer (Hewick et al., 1981). The proteins were sequenced in the presence of the carrier polybrene (Aldrich Chemical Co.)

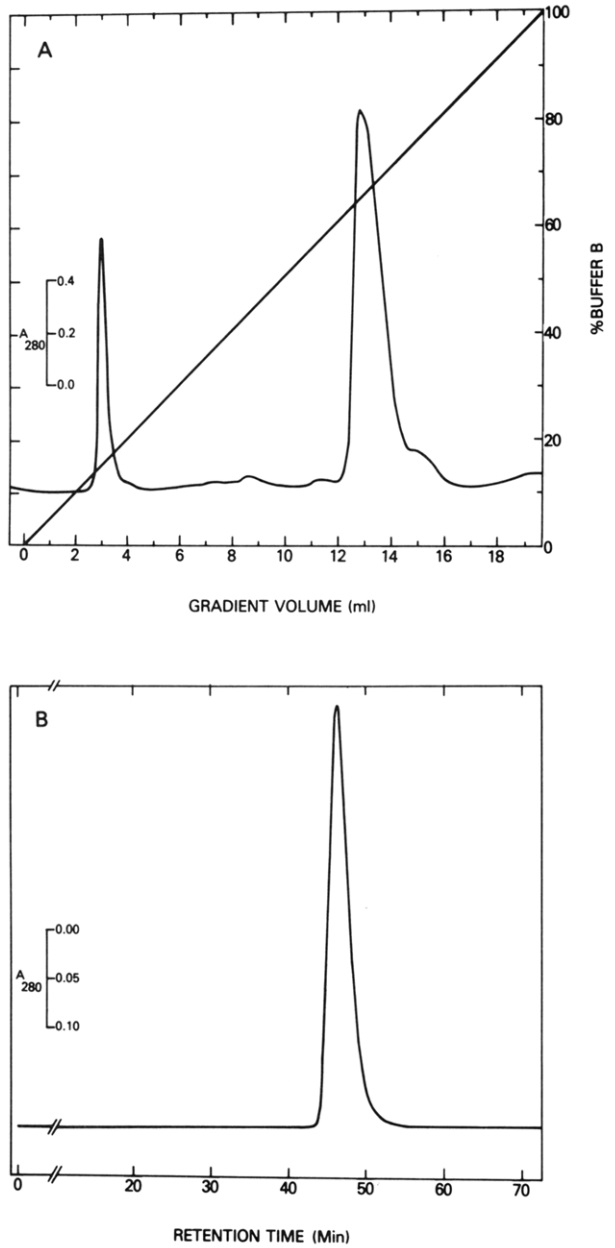

FIGURE 1: High-performance liquid chromatographic purification of monkey IRBP. (A) High-performance ion-exchange chromatography of IPM glycoproteins eluted from Con A–Sepharose batch chromatography, using a Mono-Q (Pharmacia) anion-exchange column. Conditions were as described under Experimental Procedures. Typically, half of the entire Con A–Sepharose eluate from the 30-mL IPM wash was applied. The IRBP protein peak eluted at a gradient volume of 13 mL, equivalent to a salt concentration of 0.33 M NaCl. (B) High-performance size-exclusion chromatography of the monkey IRBP protein peak from the anion-exchange step (A), using Spherogel TSK 3000 SW columns. Conditions were as described under Experimental Procedures. Typically, 500-μL injections of samples were applied. The IRBP peak showed a retention time of 46 min under the described conditions.

(Tarr et al., 1978), which was previously subjected to four complete sequencing cycles. This presequencing step helped to remove contaminants that could otherwise block the free amino terminals. The PTH-amino acids were analyzed by HPLC, using a modification of the procedure of Zimmerman et al. (1977), using a Zorbax ODS HPLC column (Du Pont Co.) (25 cm × 4.6 mm), at 50–52 °C and a linear gradient system. The gradient used was 22% solvent B increasing to 42% solvent B over 21 min at a flow rate of 1.4 mL/min. Solvent A was 0.05 M sodium acetate, pH 4.8; solvent B was

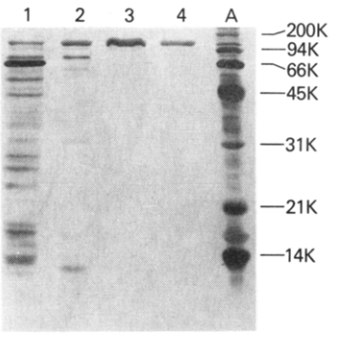

FIGURE 2: SDS–PAGE (16%) of fractions from the different stages of the purification of monkey IRBP: lane 1, crude monkey IPM wash; lane 2, methyl α-D-mannoside eluate of Con A–Sepharose column; lane 3, peak from ion-exchange chromatography; lane 4, pool of final purified IRBP preparation from size-exclusion chromatography; lane A, molecular weight markers. The gel was silver-stained. Conditions were as described under Experimental Procedures.

100% acetonitrile (Wang et al., 1982).

Spectral Properties: Tryptophan and Sulfhydryl Content. After desalting by gel filtration on PD10 columns (Pharmacia), the molar extinction coefficients of bovine and monkey IRBP were measured in water and in 6 M guanidine hydrochloride in 50 mM phosphate buffer, pH 7.0, by using a Beckman Model 25 spectrophotometer. The molar concentration of tryptophan in both these proteins was estimated by the method of Edelhoch (1967) using the proteins dissolved in 6 M guanidine hydrochloride in 50 mM phosphate buffer, pH 7.0. The same solution of protein was used to assay for total sulfhydryl group content by the method of Janatova et al. (1968).

RESULTS

Purification of IRBP. The gentle retinal wash technique for obtaining soluble components of the interphotoreceptor matrix first described by Adler & Severin (1981), was an important step in obtaining an initial preparation containing the extracellular IRBP, which is relatively free of major contamination by intracellular retinal proteins. The glycoprotein nature of IRBP afforded a further means of rapidly separating this protein from many other proteins of the IPM wash by batchwise affinity chromatography on a Con A–Sepharose gel column, but further purification was required to achieve homogeneity. The Con A–Sepharose eluate, therefore, was subjected to ion-exchange HPLC followed by size-exclusion HPLC, giving a relatively rapid purification (Figure 1). Figure 2 shows the silver-stained 16% SDS–PAGE gel of aliquots from the different stages of the purification. Lane 4 represents a sample of the IRBP preparation obtained following size-exclusion HPLC of the peak fractions from the ion-exchange HPLC step (lane 3). This preparation was homogeneous with a single band at M_r 146K. The major benefit of the ion-exchange HPLC step was to remove an unknown 14K contaminant, seen in the Con A–Sepharose eluate (lane 2), that otherwise copurified with the IRBP on size-exclusion HPLC (data not shown). Following this procedure, approximately 30 μg of monkey IRBP was recovered per mL of IPM wash. All IRBP preparations used for further analysis were monitored for homogeneity by the sensitive silver-staining method.

Bovine IRBP was purified in the same manner (data not shown). All bovine IRBP samples were also checked by silver staining of SDS–PAGE gels to ensure their homogeneity. Monkey and bovine IRBP had a comparable "apparent" M_r as assessed by SDS–PAGE.

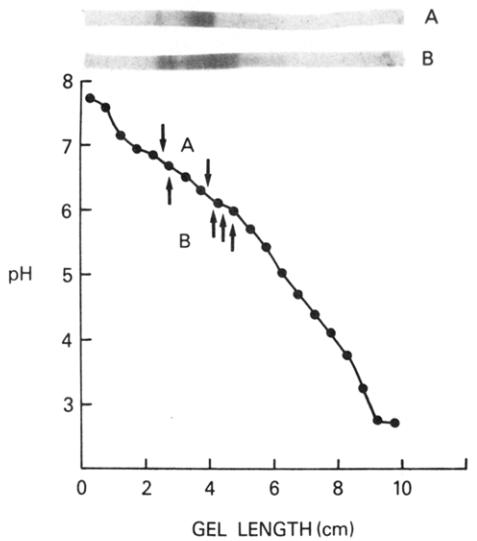

FIGURE 3: Analytical isoelectric focusing of (A) purified bovine IRBP and (B) purified monkey IRBP. The gels were focused for 16 h at 10 °C at a constant voltage of 200 V. The gels were stained as described under Experimental Procedures. The pH gradient was obtained by measurement of the pH of an overnight elution in 1 mL of water of sequential 5-mm slices of a blank gel. The arrows at (A), above the curve, indicate the major p*I* values of bovine IRBP, while those at (B), below the curve, indicate the major p*I* values of monkey IRBP.

Analytical Isoelectric Focusing. The isoelectric focusing patterns of monkey and bovine IRBPs showed considerable microheterogeneity (Figure 3). The bovine pattern exhibited about four bands with major bands at 6.3 and 6.8. The purified monkey IRBP pattern showed about eight bands with major bands occurring at 6.0, 6.1, 6.2, and 6.7.

Carbohydrate Analysis. The neutral sugar content of monkey IRBP was 18.4% if based on galactose standards and 20.2% if based on mannose. The neutral sugar content of bovine IRBP was found to be 7% based on galactose standards. The sialic acid content of bovine IRBP was estimated to be 10 mol of sialic acid/mol of protein. Fucose was detected, but not quantified, by the method of Dische & Shettles (1948).

Ultracentrifugational Analyses. A compositional partial specific volume of 0.736 cm^3/g was obtained for the polypeptide portion of the molecule. Assuming the sugar content as 20 ± 2% and the sugar density as 1.580 ± 0.40 g/cm^3, we calculated a mean corrected partial specific volume of 0.715 ± 0.005 cm^3/g. These deviations indicate probable ranges rather than standard errors.

It was found that the three simultaneously obtained ultracentrifugal concentration distributions could be simultaneously fit with single exponential distributions having the form

$$c_{r,i} = c_{b,i} \exp[AM(r_i^2 - r^2_{b,i})] + \epsilon_i \qquad (1)$$

where the c's refer to concentrations at radial positions r in the cell and r_b at the cell bottom, the i's refer to cell number (1, 2, or 3), $A = (1 - \bar{v}\rho)\omega^2/(2RT)$, $\bar{v}$ = partial specific volume, ρ = solution density, ω = rotor angular velocity, R = gas constant, T = absolute temperature, M is the molecular weight, and ϵ is a scanner base line error term. Thus, $c_{b,i}$ and ϵ_i are fitting parameters unique to each cell, and M is a fitting parameter common to all three cells and represents a mean value of the molecular weight for three determinations that are identical in all respects except for initial loading concentration.

Attempts to fit the distributions with the sum of two exponentials gave a zero concentration for the second component, thus indicating homogeneity and the absence of any self-association to form higher aggregates. A molecular weight of 106 000 ± 1000 was obtained by using the partial specific volume of 0.715 cm^3/g, the error being the standard error of M as a fitting parameter. The estimated variations in partial specific volume give a range of ±1900, resulting in a summed uncertainty of ±2900 in the molecular weight. The excellent fit to the data points and the small deviation of the data points about the fitting lines can be seen in Figure 4.

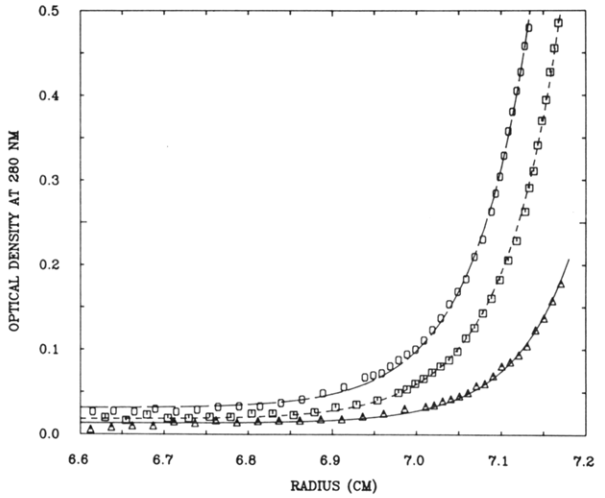

FIGURE 4: Ultracentrifugal equilibrium concentration distributions of three samples of IRBP run simultaneously for 90 h at 12 000 rpm and 5 °C. Initial concentrations were 0.057 (△), 0.114 (□) and 0.171 (○) absorbance density unit at 280 nm. The data were fitted simultaneously for the molecular weight (106 000 for $\bar{v}$ = 0.715 cm^3/g), and the fitting lines shown are for that value. The root mean square error of the combined fit is 0.005 absorbance unit.

Table I: Amino Acid Composition of IRBP from Monkey and Bovine Retina

amino acid	monkey (mol %)	bovine (mol %)	amino acid	monkey (mol %)	bovine (mol %)
Asp	6.7	7.3	Met	1.6	1.8
Thr	5.3	5.8	Ile	4.0	3.9
Ser	7.4	6.9	Leu	11.0	6.9
Glu	12.0	12.5	Tyr	2.7	3.5
Pro	5.9	6.0	Phe	2.9	3.0
Gly	11.0	9.1	Lys	3.5	3.6
Ala	9.0	10.0	His	3.9	3.1
$^1/_2$-Cys	0.5	0.8	Arg	4.3	5.9
Val	8.3	10.0	Trp		

The sedimentation coefficient (Svedberg & Pedersen, 1940) of monkey IRBP, following correction for solvent viscosity and density, was found to have a value of $s^0_{20,w}$ = 5.4 ± 0.3 S. Calculation of the frictional ratio from the values of M, $s^0_{20,w}$, and $\bar{v}$ gave a value of f/f_0 = 1.59, corresponding to an axial ratio of 11:1 for an unhydrated molecule or a lesser axial ratio depending on the extent of hydration.

Amino Acid Analysis. The amino acid compositions of monkey and bovine IRBP are given in Table I. Values are given as mole percentages.

Spectral Properties: Tryptophan and Sulfhydryl Content. The ϵ_{280} in water of monkey IRBP was 75 106 M^{-1} cm^{-1} while that of bovine was 96 836 M^{-1} cm^{-1}. The number of moles of tryptophan per mole of protein was calculated to be 8 for monkey and 10 for bovine IRBP. Standard solutions of *N*-acetyl-L-tyrosinamide and *N*-acetyl-L-tryptophanamide (Sigma) in 6 M guanidine hydrochloride were employed as models for the UV absorption spectra of the IRBPs. The number of free sulfhydryl groups in the IRBPs was calculated to be 2.73 per molecule of the monkey IRBP compared to 2.63 per molecule of the bovine IRBP.

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                          5                  10                  15
MONKEY    Gly-Pro-Thr-Ala-Leu-Phe-Gln-Pro-Ser-Leu-Val-Leu-Asp-Met-Ala

                                   5                  10                  15
BOVINE                    Phe-Gln-Pro-Ser-Leu-Val-Leu-Glu-Met-Ala-Gln-Val-Leu-(Leu)-Asp
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FIGURE 5: NH_2-terminal analysis of monkey and bovine IRBP. The underlined residues are homologous for both proteins.

Table II: Amino-Terminal Sequence Analyses of Bovine and Monkey IRBP

		bovine[a]		monkey[a]	
cycle	residue	amino acid	nmol	Gly	nmol
1	1	Phe	0.62	Gly	0.87
2	2	Gln	0.59	Pro	0.72
3	3	Pro	0.53	Thr	0.70
4	4	Ser[b]		Ala	0.76
5	5	Leu	0.50	Leu	1.09
6	6	Val	0.53	Phe	0.67
7	7	Leu	0.51	Gln	0.88
8	8	Glu	0.46	Pro	0.92
9	9	Met	0.31	Ser	0.44
10	10	Ala	0.47	Leu	0.93
11	11	Gln	0.29	Val	1.04
12	12	Val	0.54	Leu	1.03
13	13	Leu	0.55	Asp	0.62
14	14	(Leu)	0.58	Met	0.45
15	15	Asp	0.32	Ala	1.07

[a] The average repetitive yield for each cycle is 96 ± 2% for bovine IRBP and 97 ± 2% for monkey IRBP. [b] As dehydro derivative.

Amino-Terminal Analysis. The amino-terminal analyses are given in Figure 5. Table II shows the actual recovery in nanomoles of PTH from each cycle of degradation. These data confirm the purity of the preparations as assessed by silver staining of SDS–PAGE gels. If one compares residues 6–15 of the monkey IRBP with the respective residues 1–10 of the bovine IRBP, there is significant sequence homology (Figure 5). There is but one minor and conservative substitution in this sequence, i.e., Asp at residue 13 of the monkey to Glu at residue 8 of the bovine protein.

Fluorescence Studies. Figure 6 gives the excitation and emission fluorescence spectra (uncorrected) of *all-trans*-retinol complexed with monkey IRBP. The excitation λ_{max} of the bound retinol is 333 nm with emission at 470 nm. The emission λ_{max} of the bound retinol is 470 nm with excitation at 340 nm. This is blue-shifted from the emission λ_{max} of pure retinol in hexane and similar to that observed for bovine IRBP (Liou et al., 1982) as well as for cellular retinol-binding protein (Saari et al., 1978) and serum retinol-binding protein (Peterson & Rask, 1971). Further quantification of the retinoid binding functions of IRBP is under investigation.

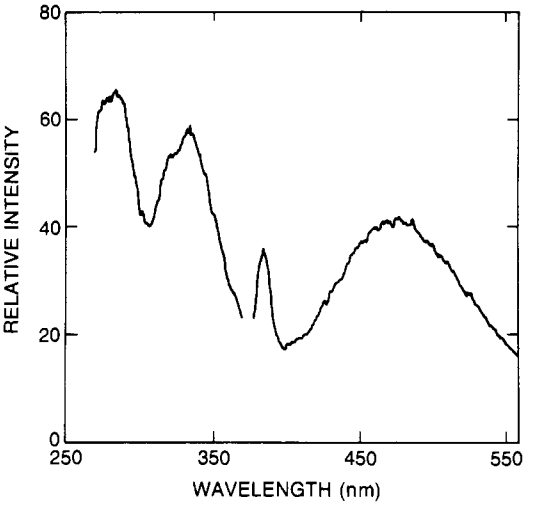

FIGURE 6: Emission and excitation fluorescence spectra (uncorrected) of *all-trans*-retinol complexed with monkey IRBP. An aliquot of eluate from ion-exchange chromatography of the Con A–Sepharose eluate was incubated with 8×10^{-7} M *all-trans*-retinol prior to size-exclusion HPLC. The emission λ_{max} was at 470 nm with excitation at 340 nm, while the excitation λ_{max} was at 333 nm with emission at 470 nm.

Discussion

The present purification technique is relatively simple because IRBP is an extracellular soluble protein that may be obtained by a gentle "washing" of whole retinas that eliminates substantial contamination by intracellular proteins. Also, its glycoprotein nature allows for further purification by affinity chromatography that removes many, but not all, other proteins. The most reliably pure samples of IRBP, as assessed by silver staining of gels following SDS–PAGE, were produced by ion-exchange HPLC of the Con A–Sepharose eluate followed by size-exclusion HPLC. Coomassie Blue staining was not sensitive enough to demonstrate the presence of a low M_r (~14K) impurity in some, but not all, samples that were obtained by size-exclusion HPLC of the Con A–Sepharose eluate.

The native molecular weight of IRBP as measured by gel filtration techniques has been reported as 250 000 in monkey retina (Pfeffer et al., 1983) and between 248 000 and 290 000 in bovine retina (Adler & Martin, 1982; Liou et al., 1982; Chader et al., 1983). SDS–PAGE has yielded values of 146 000 for monkey IRBP (Pfeffer et al., 1983) and between 140 000 and 145 000 for bovine IRBP (Liou et al., 1982; Chader et al., 1983). On the basis of the present sedimentation equilibrium analysis, we have assigned a value of 106 000 ± 2900 for the molecular weight of monkey IRBP, using an approximate carbohydrate content of 20% and a mean corrected partial specific volume of 0.715 ± 0.005 cm^3/g. The large discrepancy observed between the molecular weight of the native protein as determined by gel filtration and that determined by sedimentation equilibrium analysis may be accounted for by the asymmetric nature of the monkey IRBP as revealed by sedimentation velocity analysis. Although the contribution of hydration is undetermined, the frictional ratio of $f/f_0 = 1.59$ corresponds to an axial ratio of 11:1 for an unhydrated molecule or a lesser axial ratio depending on the extent of hydration, suggesting an elongated molecule of the prolate ellipsoid type (Svedberg & Pedersen, 1940). The difference between the SDS–PAGE and the sedimentation equilibrium values may also be accounted for, in part, by the glycoprotein nature of IRBP. It is known that glycoproteins tend to have artifactually high molecule weights on SDS–PAGE (Segrest & Jackson, 1972). In this regard, Rodbard (1976) discusses problems associated with the determination of molecular weights using gel filtration and gel electrophoresis. In addition, the sedimentation velocity analysis using the purified protein has provided a new, more accurate value of 5.4 ± 0.3 S for the sedimentation coefficient of monkey IRBP. The previously obtained value of 7 S (Wiggert et al., 1976) was calculated by using sucrose density gradient centrifugation with crude samples.

Both monkey and bovine IRBPs exhibited multiple bands in their isoelectric focusing patterns. Such microheterogeneity is commonly found in glycoproteins and is thought to be due

to differences in the carbohydrate content or type from one molecule of a particular glycoprotein to another (Williamson et al., 1973). Preliminary studies of the carbohydrate composition of the monkey and bovine IRBPs indicate the presence of neutral sugars including fucose in these proteins. Monkey IRBP had a higher neutral sugar content than the bovine protein. It was not possible to determine sialic acid in the monkey IRBP due to the scarcity of the protein, but the bovine protein had 10 mol of sialic acid/mol. Hexosamines were not detected by the amino acid analysis method used but are thought to be present based on [^{14}C]glucosamine incorporation experiments (Wiggert et al., 1984).

The amino acid compositions of the monkey and bovine IRBPs, while not identical, share many similarities. It is evident that an unusually large proportion of the residues, about 50%, are nonpolar. The strong tendency of IRBP to adsorb to glass or plastic, coupled with its extracellular matrix location, may be indicative of another function of IRBP, namely, to act as an extracellular retinal adhesion substance. It is interesting to note that McClain & Edelman (1982) have described a neural cell adhesion molecule (N-CAM) present in membranes of several neuronal cells. A similar protein has been found to be synthesized by chick retina; it has a M_r of about 140 000 and is released into culture medium in in vitro retinal incubations (Thiery, 1977) much as newly synthesized IRBP is released into the medium during incubations of intact monkey retina (Wiggert et al., 1984). We are presently investigating the possibility that IRBP may have a function similar to that of this family of proteins.

A direct comparison of the NH_2-terminal sequences of the monkey and bovine IRBPs, showing no significant homology, obscures the almost complete homology obtained when one transposes the sequences and compares residues 1–10 of the monkey and residues 6–15 of the bovine protein. Such similarities must reflect the presence of a high degree of structural homology between the monkey and bovine proteins since Bunt-Milam & Saari (1983) have reported that antiserum to bovine IRBP cross-reacts with monkey IRBP. Additionally, we have found that rabbit antiserum prepared against monkey IRBP cross-reacts with bovine IRBP (unpublished observation). With this significant homology, amino acid composition similarity, and immunological cross-reactivity in mind, it is probable that IRBP is a highly conserved protein.

IRBP thus appears to be a unique, soluble protein of the retinal interphotoreceptor matrix. It binds endogenous retinol as well as exogenously added [^{3}H]retinol (Wiggert et al., 1977) and demonstrates interesting light/dark differences in ligand binding (Wiggert et al., 1979; Lai et al., 1982; Liou et al., 1982; Adler & Klucznick, 1982) as one might expect of a putative retinoid transport protein involved in the visual process (Chader, 1982). Its presence in the interphotoreceptor space, (Pfeffer et al., 1983; Bunt-Milam & Saari, 1983) and the lack of other retinol-binding proteins (e.g., cellular retinol-binding protein, CRBP) in the monkey IPS (Pfeffer et al., 1983) further indicate a possible role for this protein as a transport vehicle in the visual process. Its location, properties, and similarities to the N-CAM protein, however, may indicate that IRBP could play a more generalized role in extracellular matrix reactions in the interphotoreceptor space.

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Effect of Capping Protein on the Kinetics of Actin Polymerization†

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ABSTRACT: *Acanthamoeba* capping protein increased the rate of actin polymerization from monomers with and without calcium. In the absence of calcium, capping protein also increased the critical concentration for polymerization. Various models were evaluated for their ability to predict the effect of capping protein on kinetic curves for actin polymerization under conditions where the critical concentration was not changed. Several models, which might explain the increased rate of polymerization from monomers, were tested. Two models which predicted the experimental data poorly were (1) capping protein was similar to an actin filament, bypassing nucleation, and (2) capping protein fragmented filaments. Three models in which capping protein accelerated, but did not bypass, nucleation predicted the data well. In the best one, capping protein resembled a nondissociable actin dimer. Several lines of evidence have supported the idea that capping protein blocks the barbed end of actin filaments, preventing the addition and loss of monomers [Cooper, J. A., Blum, J. D., & Pollard, T. D. (1984) *J. Cell Biol. 99*, 217–225; Isenberg, G. A., Aebi, U., & Pollard, T. D. (1980) *Nature (London) 288*, 455–459]. This mechanism was also supported here by the effect of capping protein on the kinetics of actin polymerization which was nucleated by preformed actin filaments. Low capping protein concentrations slowed nucleated polymerization, presumably because capping protein blocked elongation at barbed ends of filaments. High capping protein concentrations accelerated nucleated polymerization because of capping protein's ability to interact with monomers and accelerate nucleation.

Acanthamoeba capping protein is a protein which blocks the barbed end of actin filaments, binds to filaments, decreases the filament length distribution, and is not sensitive to calcium (Cooper et al., 1984; Isenberg et al., 1980). The protein also decreases the low-shear viscosity of actin filaments, an activity which initially defined the material (MacLean-Fletcher & Pollard, 1980). The protein is a heterodimer of two subunits, with a native molecular weight of 74 000, and is concentrated in the cortex of *Acanthamoeba*, where actin filaments are found (Cooper et al., 1984). These properties suggest that capping protein may function in cells to control filament length distribution, cytoplasmic viscosity, and filament location. A very similar protein has been discovered in vertebrate brain (Kilimann & Isenberg, 1982).

Actin polymerization and depolymerization may be an essential process in cell motility. In light microscopy of living *Acanthamoeba* and other motile cells, the hyaline ectoplasm, which is rich in actin filaments (Taylor et al., 1980; Pollard et al., 1970), changes its shape and moves about the cell on a rapid time scale. These changes could be due either to the movement of whole actin filaments from one place in the cell to another or to the disassembly of filaments in one place and assembly in another place. The time scale of these changes is probably too fast to allow for the diffusion of actin filaments, because by fluorescence photobleaching actin filaments in cells and at high concentrations in vitro are immobile (Wang et al., 1982; Tait & Frieden, 1982; Lanni et al., 1981). The filaments might be moved intact by bulk cytoplasmic flow. Actin monomers can diffuse rapidly, suggesting that disassembly and assembly may occur. In cells, the free monomer concentration is the critical concentration. However, at this concentration, on the basis of in vitro studies of actin, nucleation of polymerization is thermodynamically unfavorable and kinetically slow. A cell might therefore control filament formation by the use of a nucleating agent locally in the cytoplasm.

Several proteins, including villin (Craig & Powell, 1980), platelet gesolin (Kurth et al., 1983), and plasma ADF (Harris & Weeds, 1983), with activities similar to that of capping protein have been found to accelerate actin polymerization from monomers, and several possible explanations of this phenomenon have been proposed. In the experiments reported here, we analyzed the effect of capping protein on the kinetics of actin polymerization and used quantitative modeling studies to learn how capping protein affects polymerization. Capping proteins accelerated actin polymerization, and modeling studies showed that the data were consistent with capping protein acting as if it were a nondissociable actin dimer in a model where the nucleus was a trimer.

Certain preliminary experiments, which are not presented here, were presented at the 1981 Meeting of the American

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