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Expression, characterization and engineered specificity of rat epididymal retinoic acid-binding protein

Manickavasagam SUNDARAM*, Asipu SIVAPRASADARAO†[1], Daan M. F. VAN AALTEN* and John B. C. FINDLAY*

*School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K., and †Department of Pharmacology, University of Leeds, Leeds LS2 9JT, U.K.

Epididymal retinoic acid-binding protein (ERABP) is the major androgen-dependent protein present in the lumen of the epididymis and is thought to be involved in sperm maturation. It displays a high degree of three-dimensional structural similarity to serum retinol-binding protein (RBP). Although both proteins interact with retinoids, RBP exhibits a broad specificity, binding retinol, retinoic acid and retinaldehyde with roughly equal affinities, whereas ERABP is specific for all-*trans*- and 9-*cis*-retinoic acids. Consistent with this, the binding pockets of the two proteins are different: in RBP it is predominantly hydrophobic, whereas that for ERABP is amphipathic, with a network of charged residues at the open end of the binding pocket. In order to investigate the roles of these charged residues, Arg-80 and Glu-63 have been mutated to isoleucine. The resultant double mutant, Glu-63 → Ile/Arg-80 → Ile, as well as the wild-type protein, were subsequently expressed in *Escherichia coli* as fusion proteins, with the streptavidin recognition sequence (Strep) tagged to their C-termini. The expressed proteins were purified in a single step by streptavidin-affinity chromatography and their ligand-binding properties were examined using fluorimetric titrations. Whereas the wild-type ERABP binds only retinoic acid, the double mutant is capable of binding retinol, retinoic acid and retinaldehyde with similar affinities. These observations provide experimental support for the proposition that the charged residues near the open end of the binding pocket are responsible for restricting the specificity of ERABP for retinoic acid. These studies demonstrate that changes in specificity can be engineered into lipocalins.

INTRODUCTION

Vitamin A plays a critical role in sperm development (reviewed in [1]). After spermatogenesis in the testis, mammalian sperm move through the epididymis where they acquire motility. Within the lumen of the epididymis there exists a protein, termed epididymal retinoic acid-binding protein (ERABP), which is up-regulated by testosterone and is not found outside the reproductive system [2–5]. There is also some evidence to suggest that ERABP binds to sperm [1]. For these reasons, it is suggested that this protein might play a key role in sperm maturation; one possibility is that ERABP is responsible for the delivery of retinoic acid to the sperm in much the same way as has been proposed for serum retinol-binding protein (RBP) [6].

ERABP is an 18.5 kDa protein which resembles the intracellular retinoic acid-binding protein (CRABP) [7] with respect to its ability to bind all-*trans*- and 9-*cis*-retinoic acids, but not retinol and retinal. However, it displays no sequence or structural similarity to CRABP. On the other hand, the topology of ERABP [8] is very similar to that of RBP [9] and other members of the lipocalin superfamily. The characteristic feature of members of this family is that they are made of 8–9 β-strands that fold to form an orthogonal barrel [6], the inner surface of which forms the lining of the binding pocket for a hydrophobic ligand. The barrel is closed at one end and open to the medium at the other. In both RBP [9] and ERABP [8], the β-ionone ring of the ligand lies innermost at the sealed end. Consistent with this, amino acid residues situated in the closed end of the barrel are conserved between the two proteins. There is, however, substantial variation at the open end: in RBP it is predominantly hydrophobic, whereas in ERABP there is a cluster of charged amino acid residues. Thus the binding site of ERABP, like the ligand retinoic acid, is amphipathic.

As part of a study to change the molecular recognition properties of lipocalins, we have engineered the open end of the binding pocket of ERABP by replacing the charged residues with neutral amino acids. We demonstrate that simultaneous replacement of arginine and glutamic acid residues with isoleucine destroys the ability of ERABP to discriminate between retinoic acid and other retinoids, conferring the additional ability to bind retinol and retinal. These studies were performed on proteins expressed and purified from *Escherichia coli*.

MATERIALS AND METHODS

Materials

N-Acetyl-L-tryptophanamide, streptavidin–agarose, 2-iminobiotin, all-*trans*-retinol, all-*trans*-retinal, all-*trans*-retinoic acid and Lipidex-1000 were purchased from Sigma Chemical Company. Restriction endonucleases and T4 DNA ligase were obtained from Boehringer-Mannheim. *Pfu* DNA polymerase was from Stratagene. All other reagents and chemicals were of appropriate grade and were purchased from Sigma Chemical Company or BDH. *E. coli* XL1-Blue and TOPP2 strains were purchased from Stratagene. pASK60-Strep was obtained from Biometra Ltd. (Maidstone, Kent, U.K.). Custom-made oligonucleotides were obtained from Genosys Biotechnologies Inc. The Geneclean kit was from Anachem. The full-length cDNA encoding rat ERABP

Abbreviations used: CRABP, intracellular retinoic acid-binding protein; ERABP, epididymal retinoic acid-binding protein; LB, Luria-Bertani; OmpA, outer membrane protein A; RBP, retinol-binding protein; Strep, streptavidin recognition sequence.

[1] To whom correspondence should be addressed (e-mail a.sivaprasadarao@Leeds.ac.uk).

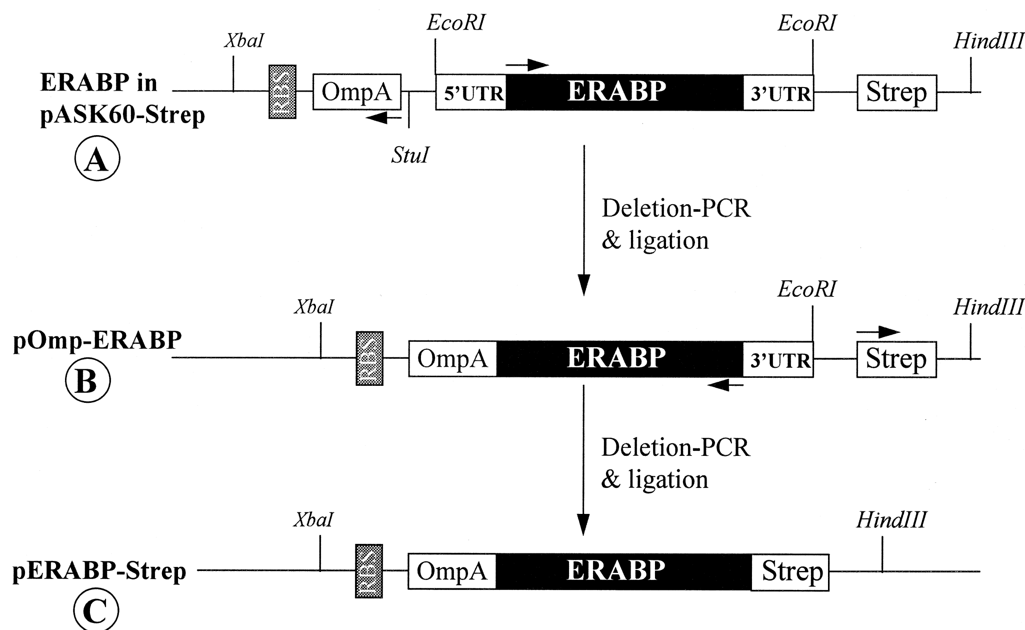

Figure 1 Schematic representation of the construction of a vector for expression of the ERABP–Strep fusion protein

5′UTR and 3′UTR are untranslated regions at the 5′ and 3′ ends of the cDNA respectively. Positions of primers used for deletion PCR are shown as horizontal arrows. For details see the Materials and methods section. RBS, ribosome binding site; OmpA, signal sequence of bacterial outer membrane protein A.

was kindly provided by Dr. L. Hall (Department of Biochemistry, School of Medical Sciences, Bristol, U.K.) in the vector pUC13 as a 652 bp *Eco*RI fragment.

Construction of a vector for ERABP expression in *E. coli*

All recombinant DNA procedures were carried out as described by Sambrook et al. [10]. A schematic diagram of the steps involved in the vector construction is shown in Figure 1. The *Eco*RI fragment containing the ERABP cDNA was first subcloned into the *E. coli* expression vector pASK60-Strep, downstream of the lac promoter between the outer membrane protein A (OmpA) signal sequence and the streptavidin recognition sequence (Strep). This construct is referred to as pASK60-ERABP–Strep (Figure 1A). This vector contains the OmpA signal sequence, which allows the expressed protein to be secreted into the periplasm, and the Strep tag, which facilitates the purification of the expressed protein by affinity chromatography using streptavidin–agarose resin. In order to fuse, in-frame, the 5′-end of the coding sequence, corresponding to the mature form of ERABP, to the 3′-end of the OmpA signal sequence, deletion PCR [11] was performed using the following primers: 5′-GCA GTG GTG AAG GAC TTC GA-3′ (sense-primer corresponding to +1 to +7 amino acid residues in ERABP) and 5′-GGC CTG CGC TAC GGT AGC-3′ (anti-sense primer representing the last six amino acid residues of the OmpA signal peptide). PCR was performed in a 100 μl volume using 10 ng of pASK60-ERABP–Strep template DNA, 200 μM deoxy-ribonucleotides, 1X buffer (supplied by the manufacturer, Stratagene), 100 pmol of each phosphorylated primer and 2.5 units of Pfu DNA polymerase. The amplification profile comprised 30 cycles, each consisting of denaturation at 95 °C for 45 s, annealing at 57 °C for 1 min and extension at 72 °C for 10 min. The resulting PCR product was purified from 1 % (w/v) agarose gel using the Geneclean kit, self-ligated at 16 °C overnight with 1 unit of T4 DNA ligase and used to transform *E. coli* XL-1Blue cells. Positive clones were identified by the loss of the first *Eco*RI site, and were later sequenced to confirm the deletion and that no errors had occurred during the PCR. A second deletion PCR was performed on the resultant construct, designated pOmp-ERABP (Figure 1B), to fuse the last codon of the EBABP coding sequence to the first codon of the Strep tag. The PCR was performed as above using the following primers: 5′-AGC GCT TGG CGT CAC CCG C-3′ (sense-primer corresponding to the first seven amino acids of the streptavidin recognition sequence SAWRHPEFGG) and 5′-GGG CCT TGA TTC AGC AGC CGA-3′ (anti-sense primer corresponding to the C-terminal seven amino acid residues of ERABP). The resultant product was purified, ligated and used to transform *E. coli* XL-1Blue cells as described above. Miniplasmid DNA isolated from the transformants were first screened for the loss of the second *Eco*RI site and later sequenced to confirm that the desired fusion had been achieved. The resulting expression vector construct, referred to as pERABP-Strep (Figure 1C), was used to express the wild-type ERABP in *E. coli*.

Construction of ERABP double mutant Glu-63 → Ile/Arg-80 → Ile

For mutagenesis, the *Xba*I and *Hin*dIII fragment encompassing the OmpA-ERABP–Strep fusion construct was isolated from pERABP-Strep and subcloned into pBluescript (KS+). Mutations were introduced into the resultant construct (pKS-ERABP) using the two-step PCR method described previously [11], with the exception that Pfu, instead of Taq DNA polymerase, was used. The Arg-80 → Ile mutation was first introduced using the M13 forward and reverse primers, and the mutagenic oligonucleotide primer, 5′-TT CAG GTC ACC A<u>TC</u> CTA CTA TCA GGA AAG-3′(the substituted bases are underlined). In the first PCR, the M13 reverse primer and the mutagenic primer were used along with 10 ng of the pKS-ERABP template DNA. Each PCR cycle (30 cycles) comprised a denaturation step at 95 °C for

45 s, an annealing step at 55 °C for 90 s and an extension step at 72 °C for 90 s. The first PCR product containing the desired mutation was purified from a 1 % agarose gel using the Geneclean kit and used as the megaprimer for the second PCR, in conjunction with the M13 forward primer and the pKS-ERABP template DNA. The PCR conditions were the same as those used for the first PCR. The product of the second PCR was fractionated on a 1 % agarose gel, purified and, after restriction with *Xba*I and *Hin*dIII, subcloned into pASK60-Strep. The mutant plasmid DNA clones were sequenced to screen for mutations and to confirm that no additional changes had been introduced. The Arg-80 → Ile mutant plasmid was subjected to a second round of PCR mutagenesis to introduce the second mutation, Glu-63 → Ile, using the mutagenic oligonucleotide, 5′-CAC TGT GTG CTG ATC AAG GTT ACA GCT-3′(the substituted bases are underlined). The other aspects of this second mutagenesis were as above.

Expression and purification of ERABP

Competent *E. coli* TOPP2 cells were transformed with the expression vector constructs containing the wild-type or the double mutant (Glu-63 → Ile/Arg-80 → Ile) ERABP–Strep fusion sequences. The transformed cells were grown on Luria-Bertani (LB)/ampicillin agar plates at 30 °C. Single colonies selected from the plate were inoculated into 50 ml of LB broth containing 100 μg/ml ampicillin (LB/amp) and grown overnight in a shaking incubator at 30 °C and 200 rev./min. A 5 ml volume of the overnight culture was then used to seed 500 ml of LB/amp and allowed to grow at 37 °C and 200 rev./min until an A_{550} of 0.6 was reached (approximately 2–3 h). Expression was then induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. After incubation for 4 h at 30 °C and 200 rev./min the cells were harvested by centrifugation for 10 min at 5000 ***g*** and 4 °C. To release the expressed protein from the periplasm, the cell pellet was resuspended in 50 ml (one tenth the volume of the original culture) of 20 % (w/v) sucrose in 35 mM Tris/HCl, pH 8.0, followed by the addition, drop-wise, of 0.5 M EDTA solution to a final concentration of 10 mM. Lysozyme was then added to a concentration of 0.1 mg/ml and the cell suspension was incubated at room temperature for 30 min. The suspension was centrifuged at 10000 ***g*** for 30 min and the supernatant was concentrated by freeze-drying. The freeze-dried material was dissolved in 5 ml of equilibration buffer (100 mM Tris/HCl, pH 8.0/1 mM EDTA) and centrifuged at 10000 ***g*** for 30 min to remove any insoluble material. The clear supernatant was then loaded on to a column (10 ml syringe) containing streptavidin–agarose (5 ml) equilibrated with 10 vol. of equilibration buffer using a flow rate of 10 ml/h. All chromatographic steps were carried out at 4 °C, as described by Schmidt and Skerra [12]. The flow was stopped for 30 min after all the sample had entered the resin. After washing the column with 10 vol. of equilibration buffer, the resin-bound ERABP–Strep fusion protein was eluted using 3 vol. of elution buffer I (100 mM Tris/HCl, pH 8.0/1 mM EDTA containing 100 μM 2-iminobiotin), followed by 3 vol. of elution buffer II (100 mM Tris/HCl, pH 8.0/1 mM EDTA containing 1 mM 2-iminobiotin). Fractions containing the pure fusion protein (21 kDa) were identified by SDS/PAGE and pooled. The purified protein after SDS/PAGE was transferred on to a nitrocellulose filter [13], treated with streptavidin–horseradish peroxidase conjugate and detected by the enhanced chemiluminescence (ECL) detection kit (Amersham). The protein content of the final preparation was determined using the method of Lowry et al. [14].

Preparation of retinoic acid–ERABP holoprotein

To the purified ERABP–Strep fusion protein (0.1 mg/ml) in 10 mM Tris/HCl, pH 7.5, an ethanolic solution of all-*trans*-retinoic acid was added to 100 μM (final ethanol concentration was kept below 5 %) and the mixture was incubated in the dark for 1 h at 37 °C. The sample was then applied to a Lipidex-1000 resin (bed vol. approx. 2 ml) and the unbound retinoic acid–ERABP complex was eluted with 3 column vols. of 10 mM Tris/HCl, pH 7.5 (free retinoic acid is retained by the resin). The eluted material was freeze-dried, dissolved in water and its UV absorbance spectrum was recorded on a Beckman DU640 spectrophotometer.

Ligand-binding studies

Steady-state fluorescence measurements were performed at 22 °C using a Perkin-Elmer LS-50B spectrofluorimeter. Binding of ligands to the apo-ERABP–Strep fusion protein was determined by following the quenching of the intrinsic tryptophan fluorescence of the protein, with excitation and emission wavelengths set at 290 and 340 nm respectively [15]. To 2 ml of 2 μM apo-protein in 10 mM Tris/HCl, pH 7.4, small increments of ethanolic solutions of all-*trans*-retinoic acid, all-*trans*-retinol or all-*trans*-retinaldehyde were added and the fluorescence was measured 5 min after each addition (5 min was found to be long enough for the fluorescent signal to reach a steady-state level). For a blank, an *N*-acetyl-L-tryptophanamide solution with an absorbance at 290 nm equal to that of the protein was used. To this, ligand additions were performed as for the protein sample. Any decrease in the fluorescence intensity of the blank *N*-acetyl-L-tryptophanamide solution is not due to interaction between the ligand and *N*-acetyl-L-tryptophanamide, but due to attenuation effects of the ligand [15]. The fluorescence intensity changes of the protein samples were corrected by subtracting the changes in the blank.

The apparent dissociation constant (K_d^{app}) was determined by fitting the quenching data to a hyperbolic equation using non-linear regression with the program FigP (Biosoft):

$$Q = (Q_{max}[L])/(K_d^{app} + [L])$$

where Q is the corrected fluorescence quenching (fluorescence of protein minus fluorescence of the corresponding *N*-acetyl-L-tryptophanamide solution), Q_{max} is the calculated maximal fluorescence quenching, [L] is the ligand concentration and K_d^{app} is the calculated apparent dissociation constant. All experiments were carried out in duplicate and the values are given as means $\pm$ S.E.M.

RESULTS AND DISCUSSION

Expression of ERABP in *E. coli*

In order to express ERABP in *E. coli*, the coding sequence of the mature form of ERABP was fused, in frame, to the *in vivo*-cleavable OmpA signal sequence at the 5′-end and to the Strep sequence at the 3′-end (Figure 1). This construct, when expressed in *E. coli*, resulted in the production and subsequent secretion of the ERABP–Strep fusion protein into the periplasm. The Strep tag allowed the purification of the secreted protein in a single affinity chromatographic step on immobilized streptavidin (Figure 2, top). Figure 2, bottom (lane 1), shows that the purified protein migrates on SDS/PAGE with an apparent molecular

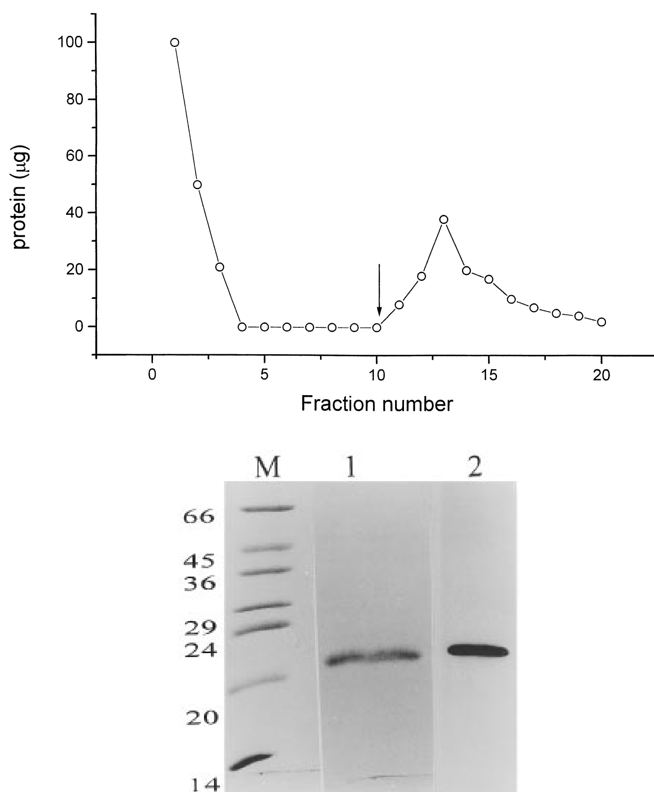

Figure 2 Purification of ERABP by streptavidin chromatography

Top: periplasmic proteins, derived from 500 ml of *E. coli* culture expressing the ERABP–Strep fusion protein, were dissolved in 0.1 M Tris/HCl, pH 8.0/1 mM EDTA, and applied to a column containing 5 ml of streptavidin–agarose resin. The elution buffer (1 mM 2-iminobiotin in 100 mM Tris/HCl, pH 8.0/1 mM EDTA) was applied at the position indicated by the arrow. Fractions 11–17 containing the fusion protein were pooled, concentrated and subjected to SDS/PAGE. Bottom: SDS/PAGE profile of the purified fusion protein (lane 1) run alongside marker proteins (Dalton Markers VII L, Sigma) of known molecular mass (lane M). A Western blot of the fusion protein obtained with streptavidin–horseradish peroxidase conjugate is shown in lane 2.

mass of 21 kDa. This is slightly heavier than the size expected from the sum of the sizes of ERABP (18.5 kDa) and the Strep tag (1.2 kDa). We interpret this as being due to anomalous migration of the fusion protein, since the N-terminal amino acid sequence of the purified protein (Ala-Val-Val-Lys-Asp), determined using automated protein sequencing, is identical with that expected for the mature protein. Thus, the OmpA signal peptide had been properly cleaved off by the bacterial signal peptidase during protein transport into the periplasm.

On Western blotting with the streptavidin–horseradish peroxidase detection system, a single band at 21 kDa (Figure 2, bottom, lane 2) was observed, indicating the presence of the C-terminal Strep tag. No other bands were detected, indicating that the protein has not suffered any degradation during purification. Since the C-terminus of ERABP is a considerable distance away from the ligand-binding site in the three-dimensional structure, we did not anticipate that the addition of the Strep tag would interfere with the ligand-binding properties of the protein. This was confirmed by the ligand-binding studies (see below). The final yield of the purified protein was about 100 μg per litre of the culture, and represented a recovery of about 30 % of the expressed protein. The poor recovery is mostly due to losses

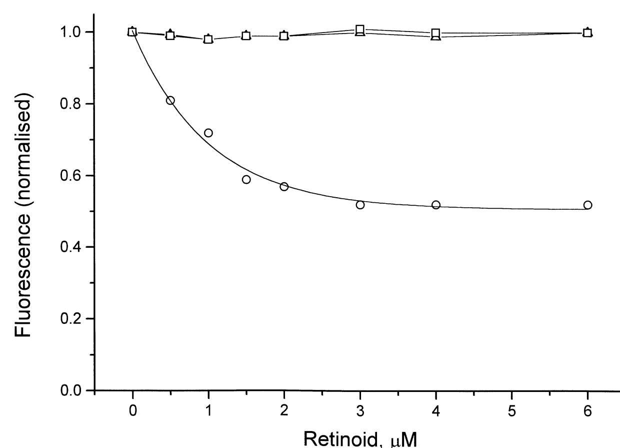

Figure 3 Fluorescence titration of the *E. coli*-derived ERABP–Strep fusion protein with retinoids

Protein fluorescence was monitored following the addition of various amounts of all-*trans*-forms of retinol (△), retinal (□) and retinoic acid (○) using excitation and emission wavelengths of 290 nm and 340 nm respectively. The data points were fitted to a mono-exponential decay equation using non-linear regression analysis. All values were corrected for a blank consisting of *N*-acetyl-L-tryptophanamide solution having a fluorescence intensity equal to that of the protein.

occurring during affinity chromatography. Our general experience is that the Strep tag system is inefficient.

Characterization of recombinant ERABP

E. coli-derived ERABP–Strep had an absorbance maximum at 280 nm and no absorbance beyond 310 nm, suggesting that the protein did not contain any bound retinoid or related chromophore. Upon reconstitution with all-*trans*-retinoic acid, the fusion protein, like native ERABP, showed an additional absorbance peak at 350 nm (due to retinoic acid). The calculated A_{350}/A_{280} ratio of the reconstituted protein was 1.25, similar to the value noted with the native protein [3]. The ability of various retinoids to bind to the recombinant ERABP was studied by following the quenching of fluorescence due to energy transfer from the single tryptophan residue of the protein to the bound ligand. The results (Figure 3) show that the all-*trans* form of retinoic acid, but not those of retinol and retinal, was capable of quenching protein fluorescence. These results, together with the UV absorbance data, indicate that the *E. coli*-expressed fusion protein has folded so as to generate a ligand-binding pocket that has the same specificity as that of the native protein. From analysis of the fluorescence quenching data, retinoic acid binds to the ERABP–Strep fusion protein with an apparent dissociation constant (K_d^{app}) of $0.9 \pm 0.2 \times 10^{-6}$ M. X-ray diffraction studies [8] suggested that ERABP contains a single molecule of retinoic acid per monomer. We have been unable to find published data on the dissociation constant for the retinoic acid–native ERABP interaction.

Site-directed mutagenesis of ERABP

A comparison of the tertiary structure of holo-ERABP [8] with that of its close homologue, serum RBP [9], suggests that the ability of ERABP to absolutely distinguish retinoic acid from retinol or retinal could be due to the charge network at the entrance to the binding pocket. This network consists of three positively charged residues (Arg-80, Lys-85 and Lys-115) and

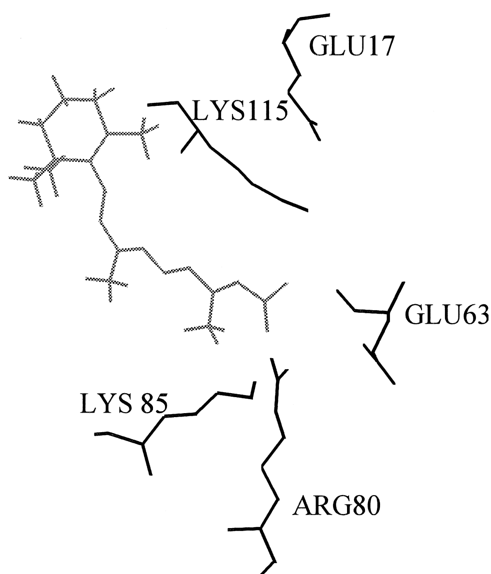

Figure 4 The charge network at the carboxylate end of the retinoic acid-binding pocket of ERABP

Retinoic acid and the relative positions of charged residues (numbered according to their position in the primary structure) near the carboxylate end of retinoic acid are shown. Coordinates used in the Figure were obtained from the Protein Data Bank, Brookhaven, NY, U.S.A.

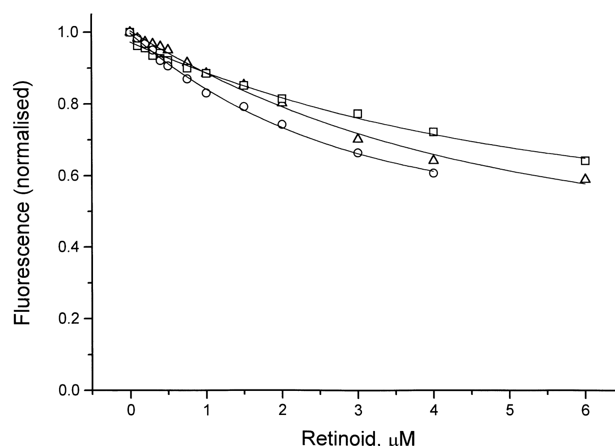

Figure 5 Fluorescence titration of the *E. coli*-derived mutant (Glu-63 → Ile/Arg-80 → Ile) ERABP–Strep fusion protein with retinoids

The titrations were performed as in Figure 3. Curves are shown for all-*trans* forms of retinol (△), retinal (□) and retinoic acid (○). No studies were carried out with the *cis*-forms.

two negatively charged amino acids (Glu-17 and Glu-63), which form an interconnecting web across the open end of the binding pocket. In the apo-form of the protein, Glu-17 and Lys-115, and Glu-63 and Arg-80, form charged pairs [8]. The side-chain of Lys-85 is located away from the binding site towards bulk solvent and is therefore unlikely to contribute to the stability or specificity of the binding pocket. Binding of retinoic acid induces conformational changes, such that in the holo-form, Arg-80 interacts with the carboxylate group of the ligand (Figure 4). Thus, Arg-80 may be responsible for restricting the ligand specificity of ERABP for retinoic acid. In order to study the role of Arg-80, a mutant ERABP was constructed in which Arg-80 was substituted with the hydrophobic amino acid isoleucine. Glu-63, with which Arg-80 forms a salt bridge in the apo-form of the protein, was also replaced with isoleucine in order to prevent any possible structural perturbations resulting from an unpaired charged residue (Glu-63) located in a hydrophobic environment. No analysis on the single mutants was performed, since crystallographic data [8] and a molecular dynamics simulation (results not shown) suggested that substitution of Arg or Glu alone might destabilize the protein.

Figure 5 illustrates that the resultant double mutant (Glu-63 → Ile/Arg-80 → Ile) is now capable of binding all the three retinoids tested, retinol, retinoic acid and retinal. Binding of all-*trans*-retinol and all-*trans*-retinal to the mutant occurred with apparent dissociation constants of $2.2 \pm 0.3 \times 10^{-6}$ M and $0.28 \pm 0.07 \times 10^{-6}$ M respectively. One explanation for this observation is that in the wild-type protein, the negative polar character of the terminal oxygen of retinol or retinal may not be sufficient to neutralize the positive charge on Arg-80, and hence the binding of these ligands is not stabilized. In the double mutant, however, where the charged residue Arg-80 is replaced with the hydrophobic isoleucine, the binding site of ERABP appears to act as a hydrophobic pocket similar to that of RBP, capable of binding retinol, retinal and retinoic acid. One interesting issue that arises from these results is that there may be subtle conformational changes which occur to accommodate ligand-binding that require Arg-80 neutralization, but which can occur readily in the absence of this charged side-chain.

The K_d of the double mutant for all-*trans*-retinoic acid ($1.6 \pm 0.2 \times 10^{-6}$ M) was not significantly different from that of the wild-type protein ($0.9 \pm 0.2 \times 10^{-6}$ M). Thus, the primary driving force for ligand-binding is likely to be the hydrophobic nature and the shape of the binding pocket, which remains the same in both the wild-type and the mutant. The charge network in ERABP appears to determine ligand specificity and/or facilitate subtle conformational changes rather than affinity. In the wild-type, the intact charge network forces the retinoic acid into a pseudo 9-*cis* conformation by 180 ° rotation about the C-8–C-9 bond [8], establishing a closer contact between the ligand and the tryptophan at the base of the binding cavity. This results in higher efficiency of energy transfer to the bound ligand and hence enhanced quenching of protein fluorescence. In the process, there are conformational changes at the mouth of the binding pocket involving charged residues. In the mutant, where the charge network is not intact, the ligand may be slightly displaced from the tryptophan, thereby accounting for decreased quenching. It would be very interesting to compare the crystallographic structures of the mutant and the native forms to establish what conformational changes have taken place.

In summary, two charged residues at the mouth of the ligand-binding pocket of ERABP are alone sufficient to confer the tight ligand-binding specificity typical of the protein. The ability to engineer the ligand specificity of the binding pockets has potential implications for the design of proteins with novel ligand- or drug-binding and drug-delivery properties. An understanding of the structure–function relationships of the binding pocket can help in the design of novel synthetic retinoids as therapeutic agents.

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