Purification and Properties of Retinoic Acid-Binding Protein from Chick-Embryo Skin

By BRAHMA P. SANI and CHANDRA K. BANERJEE

Kettering–Meyer Laboratory, Southern Research Institute, 2000 Ninth Avenue South,
Birmingham, AL 35205, U.S.A.

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Retinoic acid-binding protein, which is considered to mediate the biological function of retinoic acid in epithelial differentiation and in the possible control of tumorigenesis, was reproducibly purified from chick-embryo skin by using DEAE-Sephadex and Sephadex G-100 column chromatography and isoelectric focusing. About 1 mg of protein was isolated from 60 g of skin. The purified protein–ligand complex was found to be homogeneous by electrophoresis on polyacrylamide gels. The binding protein has mol.wt. 17800 and pI 4.5. The binding of [^3H]retinoic acid to the protein was completely inhibited by mercury compounds. The inhibition is reversible on treatment without dithiothreitol; about 50% of the retinoic acid-binding capacity of the mercury-compound-treated protein is restored by chromatography on Sephadex G-25. Iodoacetamide treatment of the protein irreversibly inhibits about 50% of retinoic acid binding.

Studies on the molecular action of retinol (vitamin A) and retinoic acid in epithelial differentiation and in the control of tumorigenesis are in progress in this laboratory. Investigations revealed the presence of a cellular retinol-binding protein (Bashor *et al.*, 1973) and retinoic acid-binding protein (Sani & Hill, 1974) that may be involved in the biological function of these retinoids and their synthetic analogues (Sani & Hill, 1974, 1976; Sani, 1975). Retinoic acid-binding protein, which was originally detected in chick-embryo skin (Sani & Hill, 1974), has since been found to be present in most of the retinoid-responsive epithelial tissues of rat, mouse and chick embryo (Ong & Chytil, 1975; Sani & Corbett, 1977), as well as in various experimental tumours and in tissues with metastatic tumour foci (Sani & Titus, 1977). It is present in the epithelial cells, but not in the connective tissues of skin (Sani & Corbett, 1977). It has been shown to have an $s_{20,w}$ value of 2.0S and a pI of 4.6 (Sani & Hill, 1974, 1976).

A retinoic acid-like structure with a ring, side chain and free carboxylic acid group is required for maximal binding to retinoic acid-binding protein (Sani & Hill, 1974). The binding affinities of retinoic acid and its synthetic analogues for the binding protein correlate with their activity in epithelial differentiation of chick-embryo skin in organ culture (Sani, 1975; Sani & Hill, 1976; Sani *et al.*, 1978). The high binding efficiency of 13-*cis*-retinoic acid and a trimethylmethoxyphenyl analogue of retinoic acid to the binding protein is in agreement with their pronounced anticarcinogenic properties (Sani & Hill, 1976; Sporn *et al.*, 1977; Sani *et al.*, 1978). Transport of retinol and retinoic acid in

plasma is facilitated respectively by a retinol-binding protein and albumin (Kanai *et al.*, 1968; Smith *et al.*, 1973).

The localization of retinoic acid-binding protein in nuclei (Sani, 1977) may be an aid in assessing the function of retinoic acid in genetic control. To understand more of this mechanism, and to understand the nature and extent of the ligand–protein interaction, a purified preparation of the protein is desirable. The present paper describes the purification and properties of retinoic acid-binding protein from chick-embryo skin and establishes that thiol functions are involved in ligand binding to this protein.

Materials and Methods

all-*trans*-[11,12- $^3\text{H}_2$]Retinoic acid was a gift from Hoffman–La Roche, Nutley, NJ, U.S.A., through the Lung Cancer Branch of the National Cancer Institute, Bethesda, MD, U.S.A. all-*trans*-[15- ^3H]Retinol was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Dithiothreitol, *p*-chloromercuribenzenesulphonic acid and iodoacetamide were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Immune γ -globulins from specific antiserum to chick serum albumin (produced in the rabbit) were purchased from Cappel Laboratories, Downingtown, PA, U.S.A. The protein-standard kit for molecular-weight determination was purchased from Boehringer–Mannheim Biochemicals, Indianapolis, IN, U.S.A. The LKB 8100 isoelectric-focusing column (110 ml) and Ampholines were purchased from LKB Instruments, Chapel Hill, NC, U.S.A.

Preparation of skin extracts

Skins (60g) from 13-day-old chick embryos were suspended in 200ml of 2mM-Tris/HCl, pH7.2, in a VirTis Teflon homogenizer, homogenized and freeze-dried. The powder was extracted with 100ml of 0.03M-sodium phosphate buffer, pH7.2, and centrifuged at 100000g for 60min. The supernatant (1950mg of protein) was adjusted to pH5.25 with 1M-acetic acid and centrifuged at 15000g for 5min. The pH of the supernatant was immediately adjusted to 7.2 with 0.5M-NaOH. This preparation was used for purification of retinoic acid-binding protein.

Studies dealing with thiol function in retinoic acid binding to its binding protein were performed by using albumin-free chick skin extract. To eliminate the albumin contamination, the extract (25mg of protein) was interacted with immunoglobulins (10mg of protein) from antiserum to chick serum albumin (Sani & Hill, 1976).

Sucrose-density-gradient sedimentation

This technique was employed to detect retinoic acid-binding protein as described previously (Sani & Hill, 1974). Portions of the [³H]retinoic acid-protein complexes at different stages of purification were layered on 5–20% (w/v) gradients of sucrose in 50mM-sodium phosphate (pH7.2) and centrifuged at 180000g for 18h by using bovine serum albumin and ovalbumin as external standards (Sani & Hill, 1976). For studies on thiol function in retinoic acid-binding protein, portions of skin extract (2.5mg of protein) were incubated for 4h with 300pmol of [³H]retinoic acid in the presence or absence of *p*-chloromercuribenzenesulphonic acid, iodoacetamide or dithiothreitol, and dialysed against 0.03M-sodium phosphate (pH7.2) containing 100mM-NaCl for 22h. The preparations were centrifuged on 5–20% linear sucrose gradients for the detection of the 2S retinoic acid-binding-protein peaks.

Purification of retinoic acid-binding protein

Extracts from 60g of chick-embryo skin after the pH5.25 isoelectric precipitation (900mg of protein) were incubated with 10nmol of [³H]retinoic acid for 4h and passed through a column (3.5cm×35cm) of Sephadex G-25 (medium grade) that was equilibrated with 0.03M-sodium phosphate buffer, pH7.2, containing 0.1mM-dithiothreitol (PD buffer). The protein eluate, which was free from unbound retinoic acid, was concentrated to 50ml by ultrafiltration with an Amicon UM 10 membrane and applied to a DEAE-Sephadex A-50 column (2.5cm×35cm) equilibrated with PD buffer. The proteins were then eluted with 100ml of the above buffer followed by 400ml of a linear gradient 0–0.3mM-NaCl in PD

buffer. The retinoic acid-binding protein-[³H]-retinoic acid-complex fractions, located by sucrose-density-gradient sedimentations, were pooled (between approx. 0.085M and 0.110M-NaCl) and concentrated by ultrafiltration on an Amicon DM5 membrane to a final volume of 10.0ml containing 91mg of protein.

The protein pool was filtered through a column of Sephadex G-100 gel (2.7cm×100cm) in PD buffer. Both protein and radioactivity distributions in the elution profile were determined. Samples (5mg each) of bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 44000), β -lactoglobulin (mol.wt. 36000), myoglobin (mol.wt. 17800), cytochrome *c* (mol.wt. 12400) and glucagon (mol.wt. 3500) were dissolved in 5ml of the elution buffer and run through the same Sephadex column as standards for molecular-weight determination. The fractions containing retinoic acid-binding protein-[³H]retinoic acid complex were pooled and ultrafiltered on an Amicon DM5 membrane to 10ml that contained 9mg of protein.

The protein pool was freeze-dried to powder, dissolved in 5.0ml of water and dialysed for 6h against 5mM-Tris/HCl, pH7.2. It was applied to a 4–48% (w/v) linear sucrose gradient on an LKB 8100 isoelectric-focusing column (110ml). Ampholines of pH3.5–7.0 were used and the anode was set at the bottom of the column. Electro-focusing was accomplished in 65h at 5°C. Fractions containing the retinoic acid-binding protein-[³H]-retinoic acid complex, which focused at pH4.5, were collected and dialysed for 16h against PD buffer. Unless otherwise mentioned, all studies were carried out at 4°C. Radioactivity measurements were made in Aquasol with a Beckman liquid-scintillation counter.

Discontinuous-gel electrophoresis

The purity of retinoic acid-binding protein was assessed on 7% polyacrylamide gels (0.5cm×12cm) at pH3.0 by using the method described by Panyim & Chalkley (1969), and at pH7.0 and 9.0 by the method described by Weber & Osborn (1969), but omitting urea and sodium dodecyl sulphate. After the protein samples had been layered, electrophoresis was carried out for 6h at 2mA/tube.

Protein concentrations were determined as described by Lowry *et al.* (1951) with bovine serum albumin as a standard. Since Ampholines also formed an intense blue colour with the Lowry *et al.* (1951) reagent, the retinoic acid-binding-protein pool after isoelectric focusing was passed through a Sephadex G-25 column (2.5cm×16cm) before protein determination. Estimation of total recovery of protein in the column fractions during purification of retinoic acid-binding protein was based on the *A*₂₈₀.

Fig. 1. Chromatographic profile of chick-embryo skin soluble protein- ^3H retinoic acid complex eluted from DEAE-Sephadex by PD buffer and a gradient of NaCl. Protein (900mg) was applied to a column (2.5cm $\times$ 35cm) and 5ml fractions were collected. The A_{280} (○) and radioactivity (Δ) distribution of the fractions are plotted. The arrow indicates where the gradient (0–0.3M-NaCl) starts.

Results

The three successive steps in the purification of retinoic acid-binding protein yielded the eluate profiles of protein distribution and radioactivity as shown in Figs. 1–3. The DEAE-Sephadex elution profile (Fig. 1) showed two radioactive peaks, material from which was characterized by sucrose-density-gradient centrifugations. The first peak, which was eluted between 0.085M- and 0.11M-NaCl, corresponded to an $s_{20,w}$ value of 2.0S and was due to retinoic acid-binding protein- ^3H retinoic acid complex. The second peak, which had an $s_{20,w}$ value of 5.0S, was due to ^3H retinoic acid bound to serum albumin, which is the transport protein of retinoic acid in plasma (Smith *et al.*, 1973; Sani & Hill, 1976). The predominant radioactive peak on the Sephadex profile (Fig. 2) corresponding to a mol.wt. of 17800 was due to retinoic acid-binding protein. The peak corresponding to the mol.wt. of about 3000 was possibly due to retinoic acid bound to small peptides. The radioactivity profile of the fractions from isoelectric focusing (Fig. 3) indicates a sharp peak at pH4.5 that corresponded to the retinoic acid-binding protein- ^3H retinoic acid complex according to its sedimentation behaviour on sucrose density gradients. Besides the principal radioactive peak, two minor unidentified peaks at pH4.8 and 5.0 were also observed.

In three preparations, starting from 60g of chick-embryo skin, the final yield of retinoic acid-binding protein was between 0.8 and 1.0mg of protein. Table 1 lists the quantitative data pertaining to the starting amount and recoveries of protein

Fig. 2. Molecular-size profiles of protein- ^3H retinoic acid complexes

The proteins obtained after DEAE-Sephadex chromatography (91mg) were resolved on a column (2.7cm $\times$ 100cm) of Sephadex G-100 gel with PD buffer. Fractions (5ml each) were collected. For molecular-weight determination, protein standards such as bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 44000), β -lactoglobulin (mol.wt. 36000), myoglobin (mol.wt. 17800), cytochrome c (mol.wt. 12400) and glucagon (mol.wt. 3500) were passed through the same column under identical conditions, and their respective elution positions are marked with arrows with their molecular weights. Symbols: Δ, radioactivity; ○, A_{280} . The position of elution of retinoic acid-binding protein (RABP) is indicated.

Fig. 3. Isoelectric-focusing profiles of fractions containing retinoic acid-binding-protein- ^3H retinoic acid complex obtained after the Sephadex G-100 step

The proteins (9mg) were electrofocused for 65h at 5°C with Ampholines of pH3.5–7.0. The pH profile (●, A_{280} (○) and radioactivity (Δ) distribution of the fractions (2ml) are plotted. The sharp radioactive peak at pH4.5 is due to the retinoic acid-binding-protein- ^3H retinoic acid complex (RABP).

and radioactivity in a typical preparation. The recoveries of total protein in each step were more than 90%. Since the protein concentrations in the fractions after isoelectric focusing were very low, the recovery of total protein could not be

Table 1. *Purification and recoveries of retinoic acid-binding protein from chick-embryo skin (60g)*

Estimation of protein recoveries was based on the A_{280} . It was not possible to estimate recovery of total protein after isoelectric focusing, because of practical problems.

	Step 1 (isoelectric precipitation at pH 5.2)	Step 2 (chromatography on DEAE-Sephadex)	Step 3 (gel filtration)	Step 4 (isoelectric focusing)
Protein at the beginning of the step (mg)	1950	900	91	9
Recovery of total protein (%)	101	92	96	—
Recovery of total radioactivity (%)	95	90	92	97
Recovery of protein in the pool (%)	46	10	10	11
Recovery of radioactivity in the pool (%)	80	56	35	31

Fig. 4. *Protein purity of retinoic acid-binding protein shown by polyacrylamide-gel electrophoresis*

A portion (5 μ g) of the purified binding protein after running in a 7% gel (0.5cm $\times$ 12cm) at pH 7.0 (see the text) in the direction indicated by the arrow.
(b) A line drawing of a.

estimated accurately. Recoveries of the binding protein in the pool from DEAE-Sephadex chromatography and Sephadex G-100 chromatography were 10% at each step: 11% of the total protein loaded on the isoelectric-focusing column was recovered in the retinoic acid-binding-protein pool.

Purity of retinoic acid-binding protein

The discontinuous-gel-electrophoretic pattern of the retinoic acid-binding-protein preparation at pH 7.0 showed a single band (Fig. 4). The preparation also migrated as a single band on electrophoresis at pH 3.0 or 9.0 (results not shown).

Thiol function in retinoic acid-binding protein

Fig. 5(a) illustrates the radioactivity-distribution profiles after sucrose-gradient sedimentation of 2.5mg of protein of chick-embryo skin extract with bound [3 H]retinoic acid, after exposure to various concentrations of *p*-chloromercuribenzenesulphonic acid. At 0.01mm- and 0.05mm-*p*-chloromercuribenzenesulphonic acid, there was a slight stimulation of [3 H]retinoic acid binding to the protein, compared with the control. About 10 and 50% inhibition of binding was observed with 0.1mm- and 0.5mm-*p*-chloromercuribenzenesulphonic acid respectively, and 1mm-*p*-chloromercuribenzenesulphonic acid completely inhibited the retinoic acid binding to the 2S protein. To learn more about the nature of the mercury-reagent-inhibited sites on the binding protein, chick-embryo skin preparations after exposure to 1mm-*p*-chloromercuribenzenesulphonic acid were further treated with 5mm-dithiothreitol followed by incubation with [3 H]retinoic acid. Sucrose-gradient-sedimentation patterns of this preparation revealed (Fig. 5b) that about 85% inhibition by mercury reagent is reversed, indicating the reversible nature of the binding sites by thiol compounds. We have also observed similar thiol function in binding of retinoic acid to its binding protein from rat testis, ovary, brain and eye (results not shown).

However, treatment with up to 2mm-iodoacetamide caused about 50% inhibition of [3 H]retinoic acid binding (Fig. 6a). Such a preparation was further dialysed in the presence of dithiothreitol. As illustrated in Fig. 6(a), no reversal of inhibition occurred in this case.

In a separate experiment, chick-embryo skin

Fig. 5. Sucrose-density-gradient profiles of *p*-chloromercuribenzenesulphonic acid (CMBS)-treated (1 h, 4°C) chick-embryo skin extract plus [^3H]retinoic acid

(a) Symbols: $\square$, control with no CMBS; $\circ$, 0.01 mM-CMBS; $\triangle$, 0.05 mM-CMBS; $\blacktriangle$, 0.1 mM-CMBS; $\bullet$, 0.5 mM-CMBS; $\blacksquare$, 1 mM-CMBS. (b) Symbols: $\triangle$, control with no CMBS; $\circ$, 1 mM-CMBS; $\bullet$, same as $\circ$, except that there was further incubation with 5 mM-dithiothreitol at 4°C for 1 h before incubating with retinoic acid. Experimental details are described in Materials and Methods section. Fractions of volume 0.2 ml were collected.

Fig. 6. Sedimentation patterns on sucrose gradients of chick-embryo skin extract plus [^3H]retinoic acid

Before incubation with retinoic acid, the extracts were treated as follows. (a) Symbols: $\square$, non-treated control; $\bullet$, 2 mM-iodoacetamide for 2 h at 4°C; $\circ$, 1 mM-*p*-chloromercuribenzenesulphonic acid; $\triangle$, same as $\bullet$, but incubated with 5 mM-dithiothreitol for 2 h at 4°C. (b) Symbols: $\triangle$, non-treated chick-embryo skin extract after passing through a Sephadex G-25 column; $\square$, chick-embryo skin extract after incubating with 2 mM-*p*-chloromercuribenzenesulphonic acid for 1 h at 4°C and passing through a Sephadex G-25 column; $\bullet$, same as $\square$, after further incubation with 1 mM-*p*-chloromercuribenzenesulphonic acid; $\circ$, same as $\square$ after incubating with 5 mM-dithiothreitol for 1 h at 4°C.

extract was treated with 2 mM-*p*-chloromercuribenzenesulphonic acid to accomplish complete inhibition of retinoic acid binding. Part of this preparation was passed through a Sephadex G-25 column (1.5 cm $\times$ 10 cm) to separate free and loosely bound mercury reagent from the more stable retinoic acid-binding-protein-mercury reagent complex. Such a preparation, after incubation with [^3H]retinoic

acid and sucrose-gradient analysis, exhibited about 50% inhibition of the retinoic acid-binding sites (Fig. 6b). This reveals that half of the mercury-reagent-sensitive sites are weak and lose the mercury reagent on molecular sieving. Such recovery from inhibition due to Sephadex sieving can be reversed to almost complete inhibition by further treatment of the Sephadex eluate with 1 mM-*p*-chloromercuri-

benzenesulphonic acid (Fig. 6b). Also, the 50% inhibition observed in the Sephadex eluates due to tight binding of the mercury reagent can be reversed almost completely to regenerate retinoic acid-binding sites by treatment with dithiothreitol (Fig. 6b).

Discussion

This report describes the purification and some properties of retinoic acid-binding protein from chick-embryo skin. About 1 mg of the protein has been reproducibly isolated from 60 g of chick-embryo skin. The way is now open for further characterization of the binding protein and for studies pertaining to its involvement in epithelial differentiation. Production of antiserum against this protein may facilitate more sensitive detection of the protein at the cellular and subcellular levels.

From its mobility on the Sephadex gels, retinoic acid-binding protein from chick-embryo skin has a mol.wt. of 17800. The same binding protein from testes has been reported by others to have a mol.wt. of 14500 (Ong & Chytil, 1975). The retinol-binding protein from the same tissue has also been shown to have the same molecular weight (Ong & Chytil, 1975). In contrast, the retinol-binding protein isolated from rat serum has an apparent mol.wt. of 21000 (Kanai *et al.*, 1968; Peterson, 1971). Thus the retinoid-binding proteins form a class of roughly the same molecular size. Although the purified protein migrated as a single band in the absence of urea or sodium dodecyl sulphate on polyacrylamide gels at pH 3.0, 7.0 and 9.0, its homogeneous nature can be established only after confirmation by more physicochemical characteristics. The acidic nature of retinoic acid-binding protein is revealed from its pI of 4.5 in Ampholines of the pH range 3.5–7.0. Previous studies with crude extracts of chick-embryo skin and a wide range of pH had indicated that the binding protein focuses at pH 4.6. Very little precipitation owing to denaturation of the protein was observed at the isoelectric point.

Although the exact nature and extent of the retinoic acid bound to its binding protein is not known, a tight binding is inferred from the stability of the ligand–protein complex to DEAE-Sephadex, Sephadex column chromatography and isoelectric focusing. However, we have observed that after freezing and thawing three times, about 50% of the retinoic acid bound to the protein is dissociated. The radioactivity peak observed at the anode (Fig. 3) is possibly caused by free retinoic acid that is liberated from the protein–ligand complex during electrofocusing.

Inhibition studies with the *p*-chloromercuribenzenesulphonic acid indicate that thiol functions are involved in the binding of retinoic acid to its

binding protein. We have also observed involvement of similar functions in [³H]retinol binding to cellular retinol-binding protein (results not shown). These findings may be directly related to the mode of molecular action of retinoids in differentiation and tumorigenesis. Under the conditions used, 1 mM-*p*-chloromercuribenzenesulphonic acid completely inhibits the retinoic acid-binding sites on the binding protein. Unlike the organic mercurial, iodoacetamide caused only 50% inhibition of retinoic acid binding. The simplest hypothesis would be to assume that there are multiple thiol functions that facilitate retinoic acid binding to the protein of which only 50% is inhibited by iodoacetamide, whereas all are sensitive to the mercury reagent. Owing to steric hindrance, iodoacetamide may have limited accessibility to certain thiol groups.

A stoichiometric assessment of ligand binding and of mercury-reagent inhibition, as well as the titration for the number of thiol groups involved in binding, will be feasible as soon as unbound retinoic acid-binding protein has been isolated. Although the protein–ligand formation is completely inhibited by the mercury reagent, and dithiothreitol reverses this inhibition almost completely, we do not yet know whether the thiol group is directly involved in the binding of retinoic acid. The data only illustrate that interaction of thiol groups on the proteins with the mercury reagent prevents the formation of the retinoid–protein complex. The use of thiol inhibitors and thiol compounds provides a fruitful approach to study the number of thiol groups involved and the nature of their involvement in retinoid–protein interactions.

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