Purification and characterization of biotin-binding protein II from chicken oocytes

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BBP-II, the major biotin-binding protein from chicken oocytes, was purified 12000-fold with a 22% yield. The purification procedure includes butan-1-ol extraction of yolk lipids, phosphocellulose chromatography of the water-soluble proteins, DEAE-cellulose chromatography at pH 7.4 and hydroxyapatite column chromatography. Final purification was obtained by using a second DEAE-cellulose column chromatography at pH 6.0. BBP-I activity separated from BBP-II activity during elution from the first DEAE-cellulose column. Purified BBP-II was homogeneous on both polyacrylamide-gel electrophoresis and SDS/polyacrylamide-gel electrophoresis under conditions that would detect a 1% impurity. The subunit M_r , determined from SDS/polyacrylamide-gel electrophoresis was 18200 (72600 for tetramer), which compares favourably with an M_r value of 17300 (69100) calculated from the amino acid analysis. A single precipitin line formed when rabbit antiserum to the protein was directed against a crude chicken egg-yolk sample. BBP-II purified by this procedure lacked carbohydrate and phosphate, was stable indefinitely when frozen, and was quite stable at room temperature. The *N*-terminal amino acid sequence showed polymorphism at three positions in the first 23 residues and was about 45% identical with the *N*-terminal 22 residues of avidin. Antiserum to BBP-II cross-reacted with BBP-I and similar proteins in the yolk of eggs from various birds and alligator as judged by immunodiffusion and enzyme-linked immunosorbent assays. No cross-reaction was observed with chicken egg-white by either of these methods.

INTRODUCTION

A biotin-binding protein (BBP) with properties quite distinct from egg-white avidin was discovered in egg-yolk (White *et al.*, 1976) and purified to homogeneity (Meslar *et al.*, 1978). This protein was found to be a tetramer having four identical subunits of M_r 18600 each. This protein was thought to have the primary role of transporting biotin from the blood plasma to the oocyte for subsequent use by the chick embryo (Mandella *et al.*, 1978; White & Whitehead, 1987). The original purification was lengthy and had poor yields (usually < 2%). Murthy & Adiga (1984) were able to improve the yields slightly with a procedure that, after denaturation with guanidinium chloride, isolated the apoprotein. Our attempts to improve the yield by incorporating chromatofocusing chromatography, dye-ligand chromatography and hydrophobic-interaction chromatography often provided considerable purification, but the resulting protein was unstable. Thus a method for purifying the stable holo-BBP in good yields was needed.

Recently it was observed that there are two biotin-binding proteins (BBP-I and BBP-II) in chicken egg-yolk that differ in their thermal stability at high dilution in a radioligand-exchange assay (White & Whitehead, 1987). On the basis of their distribution in immature and adult chickens, the two activities appear to be functionally differentiated. BBP-I is probably involved in biotin distribution for general maintenance, a function that overlaps with that of BBP-II, which is specifically

involved in transport of biotin to the oocyte. The nature of these two activities and whether the previously purified BBP was one of these or a mixture was not known. Therefore the present studies were initiated to resolve these issues and to improve yields. In contrast with previous purifications, we used yolk from oocytes rather than eggs, thus avoiding any contamination with avidin. The present paper summarizes the purification procedure, reports some stability characteristics of the purified protein and presents immunological evidence for BBP in egg-yolk from several different species.

MATERIALS AND METHODS

Materials

The following were obtained from the indicated commercial sources: M_r markers, polyacrylamide, SDS, HTP hydroxyapatite and Tween 20 from Bio-Rad Laboratories; phosphocellulose, goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate and *p*-nitrophenyl phosphate from Sigma Chemical Co.; DE-52 DEAE-cellulose from Whatman; (MPL+TDM) adjuvant from Ribi Immunochemical Research; D-[¹⁴C]biotin (specific radioactivity 49 mCi/mmol) and aqueous counting scintillant from Amersham.

BBP assay

Total egg-yolk BBP was assayed by the heat-accelerated exchange to equilibrium of [¹⁴C]biotin with unlabelled endogenously bound biotin (White *et al.* 1987). This

Abbreviations used: BBP, biotin-binding protein; PEG, poly(ethylene glycol); PAGE, polyacrylamide-gel electrophoresis; (MPL+TDM) emulsion, monophospho-lipid A + trehalose dimycolate + squalene (hexamethyltetraacosahexane) + mono-oleate (Tween 20); e.l.i.s.a., enzyme-linked immunosorbent assay.

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assay does not distinguish between BBP-I and BBP-II. The corresponding heat accelerated exchange of [^3H]-biotin at 65 °C and at high sample dilution denatures BBP-II but not BBP-I (White & Whitehead, 1987). One unit of biotin-binding activity is equal to 1 μg of biotin bound.

Protein assay

Absorbance at 280 nm was used to monitor protein content during the chromatographic steps. Total protein was determined by using the method based on the difference in absorbance at 235 and 280 nm (Whitaker & Granum, 1980).

Purification of egg-yolk BBP-II

Homogenization and lipid extraction. Chicken ovaries were obtained fresh from Dover Poultry (Baltimore, MD, U.S.A.) and chilled on ice. The individual ovaries were cut open and the yolk from the oocytes was strained through needlepoint canvas [3 mm ($\frac{1}{8}$ in) holes], yielding 8 litres of yolk. The yolk was diluted 1:1.5 (v/v) with 1.6 M-NaCl and homogenized by stirring. Lipids were extracted and the denatured proteins were separated from water-soluble proteins by centrifugation of an emulsion formed by a mixture of the crude extract with butan-1-ol as reported elsewhere (Meslar *et al.*, 1978). From this point on, all steps were carried out at 4 °C in solutions containing 0.02 % NaN_3 .

PEG precipitation. The aqueous layer from the butanol extraction was initially made 9 % in PEG 8000 by adding 215 ml of 50.9 % (w/v) PEG to each litre of the aqueous layer, and the precipitate was allowed to settle out overnight. The next day most of the top clear supernatant was decanted and pooled. The remaining supernatant was obtained by centrifuging (13 000 *g* for 30 min) the lower turbid layer. To each litre of 9 % PEG supernatant was added 300 ml of the 50.9 % PEG solution. The resulting 19 % PEG solution was stirred and the precipitate was allowed to settle overnight. The next day the sticky 9–19 % PEG precipitate was scraped from the bottom of the container and dissolved in the minimum amount of 25 mM-Tris/HCl buffer, pH 7.4.

Phosphocellulose chromatography. The dissolved 9–19 % PEG precipitate was dialysed against 50 mM-potassium phosphate buffer, pH 6.0, and applied directly to a phosphocellulose column (5 cm $\times$ 60 cm) previously equilibrated with this buffer. After extensive washing with the above buffer to remove non-bound proteins, BBP and other bound proteins were batch-eluted with 1.0 M-NaCl in 50 mM-potassium phosphate buffer, pH 6.0. The active fractions were pooled and prepared for the next chromatographic step.

First DEAE-cellulose chromatography. The phosphocellulose eluate, dialysed against 25 mM-Tris/HCl buffer, pH 7.4, was pumped directly on to a DEAE-cellulose column (3 cm $\times$ 60 cm) equilibrated with the same buffer. After non-bound proteins were removed by extensive washing with this buffer, bound proteins, including BBP-I and BBP-II, were eluted during a linear gradient of 0–0.25 M-NaCl in 25 mM-Tris/HCl buffer, pH 7.4. Fractions containing BBP were pooled.

Hydroxyapatite column chromatography. The DEAE-cellulose eluate was dialysed against 10 mM-potassium phosphate buffer, pH 7.2, and pumped directly on to a hydroxyapatite column (2 cm $\times$ 30 cm) equilibrated with this buffer. A linear gradient of 10–500 mM-potassium phosphate buffer, pH 7.4, was used to elute BBP-II and several other bound proteins.

Second DEAE-cellulose chromatography. The pooled fractions containing BBP-II from the preceding step were dialysed against 10 mM-potassium phosphate buffer, pH 6.0, and applied to the DEAE-cellulose column (2 cm $\times$ 30 cm). After washing, the BBP was eluted with a linear gradient of 0–0.5 M-NaCl in the equilibration buffer. At pH 6.0 BBP loses activity, so the pooled active fractions were promptly dialysed against 100 mM-potassium phosphate buffer, pH 7.2, where BBP is quite stable.

Electrophoresis

PAGE (Weber & Osborn, 1969) and SDS/PAGE (Davis, 1964) were run to determine degree of purity and subunit M_r . Protein bands were stained with Coomassie Blue (Blakesley & Boezi, 1977). The M_r standards were lysozyme (14 400), soya-bean trypsin inhibitor (21 000), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (66 200) and phosphorylase *b* (92 500).

Amino acid analysis

Amino acid analyses were run on samples (8 nmol of protein) after acid hydrolysis for 24, 48 and 72 h at 110 °C (Moore & Stein, 1963). Tryptophan was determined by the method of Edelhoch (1967). A BBP-II sample was dansylated, hydrolysed, dried and redissolved in 50 % (v/v) pyridine (Woods & Wang, 1967). The *N*-terminal amino acid was identified by two-dimensional t.l.c. on a 5 cm $\times$ 5 cm polyamide sheet.

N-Terminal amino acid sequence analysis

About 4.0 nmol of pure BBP-II was dialysed against distilled deionized water, and *N*-terminal amino acid sequence analysis was performed with an Applied Biosystems 470A protein sequencer coupled with a 120A phenylthiohydantoin analyser (Wittmann-Liebold *et al.*, 1976; Hewick *et al.*, 1981).

Phosphate analysis

Analysis for phosphate was done by following the method of Ames (1966). About 1.5 mg of BBP-II was used for this determination. Bovine serum albumin was used as a negative control and serum riboflavin-binding protein was used as a positive control.

Carbohydrate analysis

About 1.5 mg of BBP-II (10 mg/ml in distilled deionized water) was analysed for the presence of carbohydrate by using g.l.c. after overnight hydrolysis in 20 mM-HCl at 100 °C (Porter, 1975). Chicken serum riboflavin-binding protein was used as a positive control.

Molar absorption coefficient

Three different methods were employed to determine the 280 nm molar absorption coefficient of BBP-II. The first was a determination of absorption coefficients of purified proteins by conventional u.v. spectrophotometry

with multi-wavelength detection (van Iersel *et al.*, 1985). Secondly, the method of Whitaker & Granum (1980), which is based on the difference in absorbance at 235 and 180 nm wavelength for determining protein concentration, was used. Finally, a 2.00 mg freeze-dried sample of pure BBP-II, previously dialysed against distilled deionized water, was dissolved in 2.0 ml of distilled deionized water, and from this known concentration and absorbance of the solution a molar absorption coefficient was determined. The values obtained by these methods were compared with one calculated from the amino acid composition (Wetlaufer, 1962).

Stability studies

The stability of BBP-II to prolonged dialysis against distilled deionized water was determined. The room-temperature-stability characteristics of BBP-II were investigated by assaying a sample of BBP-II in 50 mM-potassium phosphate buffer, pH 7.2, containing 0.02 % NaN₃ kept at room temperature (approx. 25 °C) for 2 months. Finally, stability to repeated freezing and thawing was examined.

Preparation of anti-BBP-II antibody

New Zealand White rabbits were immunized with purified chicken egg-yolk BBP-II. Before each injection the BBP-II sample was dialysed against saline solution to remove any azide present in the chromatographic buffer. The BBP-II preparation (100 µg of protein), emulsified with the (MPL + TDM) adjuvant, was injected intramuscularly, followed by two subcutaneous booster injections (75 µg and 50 µg of protein) 4 and 8 weeks later respectively. The first of these booster injections was done in the (MPL + TDM) emulsion and the last booster injection was done in saline solution alone. At 1 week following the second booster injection the rabbits were bled and the serum was collected after allowing the clotted blood to set in the refrigerator overnight. The crude serum was used without further purification.

E.I.s.a.

This procedure was based on standard e.i.s.a. protocols (Monroe, 1984; Voller *et al.*, 1976; Haid & Swissa, 1983). In summary, the coating buffer used was 50 mM-sodium carbonate buffer, pH 9.6, and the washing buffer was 145 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 0.05 % Tween₂₀. The secondary antibody was an anti-(rabbit IgG) antibody-alkaline phosphatase conjugate. *p*-Nitrophenyl phosphate in diethanolamine was used as substrate. The absorbance at 405 nm was read after various time intervals with the use of a Microelisa plate reader. Values were corrected for controls in which pre-immune serum was used. A strongly positive e.i.s.a. response was seen, the A_{405} (sample)/ A_{405} (pre-immune) ratio being greater than 5.

Cross-reactivity of anti-BBP-II antibody with similar proteins

Several experiments were conducted to test for cross-reactivity of anti-BBP-II antibody with similar biotin-binding proteins in other species. The crude egg-yolks and egg-whites (diluted 1:1.5 with 1.5 M-NaCl) of the following species were tested: chicken (*Gallus gallus domesticus*), goose (*Anser anser*), Peking duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*) and alligator (*Alligator mississippiensis*). An e.i.s.a. assay was first performed followed by an Ouchterlony immunodiffusion test.

RESULTS

Purification

A summary of the purification procedure is presented in Table 1. Fig. 1 shows the elution profile of BBP-I and BBP-II from a DEAE-cellulose column. When analysed with the [³H]biotin-exchange assay, BBP-I and BBP-II activities are clearly separated during the early part of the salt gradient. Fractions containing BBP-II activity were pooled and purified on a hydroxyapatite column

Table 1. Summary of BBP-II purification from 8 litres of chicken oocytes

For full experimental details see the text. The 40 °C/65 °C activity ratio is the ratio of biotin-binding activity after 45 min at 40 °C to that at 65 °C with the [³H]biotin-radioligand-exchange assay.

Purification stage	Volume (ml)	BBP activity (units)	Total protein (mg)	Specific activity (units/mg)	Cumulative yield (%)	Purification (fold)	40 °C/65 °C activity ratio
Diluted egg-yolk	20000	1900	1.93×10^6	1.0×10^{-3}	100	1	5.0
Butan-1-ol-extracted yolk	5100	1840	220×10^3	8.4×10^{-3}	96	8	—
9 % PEG supernatant	5900	1510	144×10^3	10.0×10^{-3}	78	10	—
9–19 % PEG ppt.	500	1400	54.0×10^3	26.3×10^{-3}	72	26	5.0
Phosphocellulose eluate	1000	990	1000	0.9	51	950	5.2
1st DEAE-cellulose eluate	100	700	140	5.0	37	5000	50
Hydroxyapatite eluate	40	520	55	9.4	27	9500	400
2nd DEAE-cellulose eluate	15	430	35	12.0	22	12000	300

Fig. 1. Elution of BBP from a DEAE-cellulose column

BBP-containing fractions eluted from the phosphocellulose column were pooled, dialysed and run through a DEAE-cellulose column (3 cm $\times$ 60 cm) that had been equilibrated with 25 mM-Tris/HCl buffer, pH 7.4. The column was washed extensively with this buffer until the A_{280} baseline was reached, then BBP-I and BBP-II were eluted during a 500 ml linear gradient of 0–0.25 M-NaCl in 25 mM-Tris/HCl buffer, pH 7.4 ($\diamond$). Fractions (2 ml) were collected at a flow rate of 2 ml/min. The [3 H]biotin-radioligand-exchange assay was used to monitor BBP activities after incubation at 40 $^{\circ}$ C for BBP-II ($\blacklozenge$) and at 65 $^{\circ}$ C for BBP-I ($\blacksquare$). Protein was monitored by the absorbance at 280 nm ($\square$). BBP-I fractions (80–98) were pooled and frozen for later purification. BBP-II fractions (100–125) were pooled and prepared for the next step.

followed by a second DEAE-cellulose column. Fig. 2 shows the elution profile of BBP-II from the hydroxyapatite column and Fig. 3 shows the elution profile from the DEAE-cellulose column at pH 6.0.

Analysis of purity

The purified protein appeared to be homogeneous on the basis of the following criteria. A single band was observed on PAGE and on SDS/PAGE. Gel scans showed only a single absorption peak corresponding to BBP-II (Fig. 4). The specific activity of the eluate from the second DEAE-cellulose column of 12 units/mg (range 10.3–12.9) approaches the theoretical value of 13.0 units/mg for pure protein, which corresponds to 1 mol of biotin bound/mol of subunit (Meslar *et al.*, 1978). Serial dilution (1–32 μ g) of BBP-II followed by SDS/PAGE and gel scans showed only a single band, with the BBP-II being at least 99% pure. *N*-Terminal amino acid analysis showed only dansyl-valine in agreement with earlier results (Meslar *et al.*, 1978). However, *N*-terminal sequencing (Table 2) shows about 30% glycine, indicating a ragged *N*-terminus. Immunodiffusion of crude chicken egg-yolk containing BBP against rabbit anti-(BBP-II) serum yielded a single precipitin line. The amino acid composition was not significantly different from that previously reported (Meslar *et al.*, 1978).

On the basis of SDS/PAGE, the estimated M_r of the BBP-II subunit is 18200 (72600 for the tetramer). This

compares well with that of 17300 (69200) calculated from the amino acid composition.

Heat-stability characteristics

Fig. 5 shows the temperature-dependence of the [3 H]biotin-exchange assay on extracts containing both BBP-I and BBP-II. The two components are resolved in Fig. 6, which shows that one component (BBP-I) is stable up to 65 $^{\circ}$ C for 45 min and the other (BBP-II) is stable at 40 $^{\circ}$ C for 45 min but denatures at 65 $^{\circ}$ C. This differentiation requires the high sample dilution. At the higher protein concentrations required in assays with [14 C]-biotin (White *et al.*, 1976) no difference is observed between assays at 45 $^{\circ}$ C and 65 $^{\circ}$ C. Increasing the protein concentration in the [3 H]biotin-exchange assay into the range used for the [14 C]biotin exchange eliminates the stability differences (L. Bush & H. B. White, III, results not shown). The exact nature of this effect is not known but may involve protein subunit dissociation and denaturation. The kinetics of the [3 H]biotin-exchange reactions at several different temperatures are shown for BBP-I (Fig. 7) and BBP-II (Fig. 8).

Molar absorption coefficient

The molar absorption coefficient of BBP-II at 280 nm was determined by three methods to be 43300 (first method), 41900 (second method) and 43600 $\text{M}^{-1}\cdot\text{cm}^{-1}$ (third method). A value of 43000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ is considered to be the best estimate with the use of M_r value of

Fig. 2. Hydroxyapatite chromatography of BBP-II

Active fractions of BBP-II pooled from the previous DEAE-cellulose column were dialysed against 10 mM-potassium phosphate buffer, pH 7.2, and pumped directly on to a hydroxyapatite column (2 cm × 30 cm) previously equilibrated with this buffer. The column was extensively washed with this buffer to remove non-bound proteins, and BBP-II (◆) was eluted during a 200 ml linear gradient of 10–500 mM-potassium phosphate buffer, pH 7.2 (■). Fractions (2.5 ml) were collected at a flow rate of 0.30 ml/min. Protein was monitored by the absorbance at 280 nm (□). BBP-II fractions (16–41) were pooled and prepared for the final step.

Fig. 3. DEAE-cellulose chromatography of BBP-II at pH 6.0

Active fractions pooled from the previous hydroxyapatite column were dialysed against 10 mM-potassium phosphate buffer, pH 6.0, and pumped directly on to a DEAE-cellulose column (2 cm × 30 cm) equilibrated with this buffer. Non-bound proteins were removed by washing with this buffer, and homogeneous BBP-II (◆) was eluted with a 100 ml linear gradient of 0–0.5 M-NaCl (■) in 10 mM-potassium phosphate buffer, pH 6.0. Fractions (1.5 ml) were collected at a flow rate of 0.5 ml/min. Protein was monitored by the absorbance at 280 nm (□). Fractions (38–45) were checked for purity by SDS/PAGE.

Fig. 4. Assessment of the purification of BBP-II by SDS/PAGE

The main Figure shows a gel scan, with a Beckman DU instrument, of the purified protein. The direction of the migration was from left to right. Inset: lane 1, M_r standards lysozyme (14400), soya-bean trypsin inhibitor (21000), carbonic anhydrase (31000), ovalbumin (45000), bovine serum albumin (66000) and phosphorylase *b* (92500); lane 2, phosphocellulose eluate; lane 3, DEAE-cellulose column eluate at pH 7.4; lane 4, hydroxyapatite column eluate; lane 5, BBP-II from the DEAE-cellulose column at pH 6.0.

Fig. 5. $[^3\text{H}]$ Biotin-exchange curve as a function of temperature in partially purified egg-yolk

A 250-fold dilution of 19% PEG precipitate material containing both BBP-I and BBP-II was incubated at various temperatures for 45 min in the presence of $[^3\text{H}]$ biotin and the bound $[^3\text{H}]$ biotin was determined.

Fig. 6. Effect of temperature on BBP-I and BBP-II $[^3\text{H}]$ biotin-exchange reactions

Samples of pure BBP-II (30 μl of a 0.05 mg/ml solution) and BBP-I (100 μl of 1/100 dilution of 19% PEG precipitate) were incubated at various temperatures for 45 min in the presence of $[^3\text{H}]$ biotin and the bound $[^3\text{H}]$ biotin was determined. The 19% PEG precipitate containing both activities was first heated at 65 $^{\circ}\text{C}$ for 1 h to denature selectively all BBP-II material present.

72000. This compares favourably with the value of $38050 \text{ M}^{-1} \cdot \text{cm}^{-1}$ calculated from the amino acid composition. The ratio of observed to calculated values of 1.13 is in the range seen for other proteins (Wetlaufer, 1962).

Presence of phosphate and carbohydrate

Two separate determinations for phosphate showed less than $1 \mu\text{mol}$ of phosphate/ μmol of subunit. The g.l.c. analysis of hydrolysed BBP-II failed to detect the presence of any carbohydrate.

Sequence analysis

The first 23 residues of the *N*-terminal amino acid sequence is reported in Table 2. A comparison with the first 22 residues of the avidin *N*-terminal sequence is also indicated.

Stability studies

Repeated freezing and thawing of the pure BBP-II kept in 50 mM-potassium phosphate buffer, pH 7.2, containing 0.02% NaN_3 did not result in any loss of activity. However, dialysing this protein sample against distilled deionized water at 4 $^{\circ}\text{C}$ for 24 h did result in some loss of activity, usually around 40–50%, although sometimes as high as 80%. When a BBP-II sample was kept in the above buffer, the protein was considerably more stable than when it was dialysed against distilled deionized water regardless of whether it was kept at 25 $^{\circ}\text{C}$ or 4 $^{\circ}\text{C}$. Finally, the stability of BBP-II, in the above buffer, for 2 months at room temperature was tested. Over the entire period there was about 25% loss of activity. However, about 20% of this loss occurred over the first 5 h.

Fig. 7. Kinetics of biotin exchange for BBP-I at 40 °C (◆) and 65 °C (◻)

The 19 % PEG precipitate was preheated as indicated in Fig. 6 legend to denature BBP-II before the addition of the [³H]biotin.

Fig. 8. Kinetics of biotin exchange for pure BBP-II (1.5 µg/ml solution) at 35 °C (◼), 40 °C (◆) and 45 °C (◻)

Characterization of antiserum to BBP-II

Rabbit antiserum to chicken egg-yolk BBP-II yielded a single precipitin line on immunodiffusion versus either purified BBP-II or diluted egg-yolk. No precipitin lines were seen when the pre-immune serum was used.

By using the e.l.i.s.a. assay, it was established that a 1/500 dilution of the crude serum containing the anti-BBP-II antibody incubated with the substrate for 10 min

Table 2. *N*-Terminal primary sequence of BBP-II

Bold face type denotes the probable amino acid residue at that position. Underlined residues are those that are identical with avidin. For alignment purposes the *N*-terminal of avidin corresponds to residue no. 2 of BBP. Residues in parentheses show heterogeneity.

Residue no.	Amino acid	Yield (pmol)
1	Val , Gly	550, 250
2	Glu , Val	510, 35
3	(Ile , Arg), Glu	230, 155, 75
4	<u>Lys</u> , Arg	420, 60
5	His , Lys	75, 60
6	Gln	510
7	<u>Leu</u> , Gln	410, 75
8	Ser , Leu	300, 65
9	(Gly , Pro), Ser	250, 180, 45
10	Leu , Gly, Pro	275, 80, 40
11	<u>Trp</u> , Leu	160, 60
12	(Arg , Glu), Trp	155, 120, 50
13	<u>Asn</u> , Arg, Glu	270, 90, 50
14	Glu , Asn	220, 75
15	Gln , Glu	250, 100
16	Asp , Gln	200, 80
17	Ser , Asp	120, 80
18	Leu , Ser	125, 40
19	Met , Leu	180, 60
20	Glu , Met	110, 65
21	<u>Ile</u> , Glu	90, 60
22	Ser , Ile	90, 50
23	<u>Ala</u> , Ser	155, 42

gives an acceptable response when tested against 1 µg of pure BBP-II.

Cross-reactivity of anti-BBP-II serum with similar proteins

It was of interest to determine if similar biotin-binding proteins are present in the egg-yolks of other oviparous species. A positive e.l.i.s.a. response was seen with the egg-yolks of each species tested, and a negative response was seen with the egg-whites, including chicken. A single precipitin line was seen when the anti-BBP-II serum was directed against the egg-yolks of the above species except alligator, in which no precipitin line was seen. No precipitin line was seen with the egg-whites of all the species tested. Table 3 summarizes the cross-reactivity studies.

DISCUSSION

Previous attempts at purifying chicken egg-yolk BBP were only partially successful. Although pure protein was obtained, it represented at best only a 1–2 % yield, and the resulting protein was invariably unstable, often losing more than 50 % of its activity in the cold, even if kept in a buffered solution. The cause of this instability is unclear, but may have been due to apoprotein formation during the various chromatographic steps. The results presented here show a greater than 10-fold improvement on yield, and the protein is considerably

Table 3. Comparison of biotin-binding proteins from bird and alligator eggs

All yolk samples were diluted 250-fold with buffer and assayed with the [^3H]biotin-radioligand-exchange assay. Incubations were at 40 °C or 65 °C for 45 min.

Sample	E.l.i.s.a. assay (A_{405})	Concn. of BBP (nM)		
		BBP (40 °C)	BBP (65 °C)	BBP (40 °C)/ BBP (65 °C) ratio
Purified chicken BBP-II (0.66 mg/ml)	0.96*	6000	20.0	300
Chicken egg-yolk	0.62	130	30.0	4.3
Peking-duck egg-yolk	0.40	70.0	140	0.5
Turkey egg-yolk	0.37	40.0	40.0	1.0
Goose egg-yolk	0.40	160	440	0.4
Alligator egg-yolk	0.26	10.0	170	0.1

* Value of pre-immune serum used as a reference negative sample was 0.06.

more stable as long as it is kept in a buffered solution at pH 7.2.

The original purifications of BBP to homogeneity were monitored with the [^{14}C]biotin-radioligand-exchange assay at 65 °C (White *et al.*, 1976; Meslar & White, 1979). When the more sensitive assay for BBP based on an equilibrium exchange of [^3H]biotin was used (White & McGahan, 1986), it was evident that we had isolated BBP-II (White & Whitehead, 1987). Re-analysis of the various partially purified samples for their [^3H]biotin exchange at 40 °C and 65 °C showed that BBP-I activity separated from BBP-II activity during the first DEAE-cellulose gradient elution (last column of Table 1 and Fig. 1).

The procedures previously used to purify BBP have involved one or more harsh steps (McGahan, 1984; Meslar *et al.*, 1978; Murthy & Adiga, 1984) and have resulted in relatively poor yields, both features which suggest that BBP-I was isolated. However, the amino acid composition of BBP-II isolated here is indistinguishable from that reported by Meslar *et al.* (1978). Thus if earlier preparations (McGahan, 1984; Meslar *et al.*, 1978) are BBP-I, they are subtly different from BBP-II.

Meslar *et al.* (1978) reported that BBP was a glycoprotein based on its staining with the periodic acid/Schiff reagent for carbohydrate. McGahan (1984) did detect carbohydrate using g.l.c. analysis, but the small amounts and stoichiometry of the sugars were not compatible with that normally found in glycoproteins (Montreuil, 1980). Our g.l.c. analysis of hydrolysates of purified BBP-II failed to detect sugars. We conclude, therefore, that BBP-II is not a glycoprotein.

The *N*-terminal sequence reported here (Table 2) was part of a run of 45 cycles. Ten of the first 23 residues of BBP-II are identical with the first 22 residues of the avidin sequence, suggesting the two proteins are homologous. This relationship was also suggested by the subunit composition and M_r (Meslar *et al.*, 1978). Avidin is the biotin-binding protein in chicken egg-white, which normally has little bound biotin, and is thought to function as an antimicrobial agent (Tranter & Board, 1982).

Further evidence that BBP-I and BBP-II are quite similar is seen in the fact that both proteins cross-react

strongly with the anti-BBP-II (as seen by the fact that both proteins have virtually the same e.l.i.s.a. response; L. Bush & H. B. White, III, unpublished work). However, the two proteins show different binding behaviours on the DEAE-cellulose column (Fig. 1) and hydroxyapatite column chromatography (L. Bush & H. B. White, III, unpublished work), suggesting a charge difference between the two. We have only recently purified BBP-I to homogeneity and found it to be a monomer with an M_r of about 69000 by SDS/PAGE. This is in direct contrast with BBP-II, which is composed of four identical subunits. However, the *N*-terminal amino acid of BBP-I was determined to be valine, in agreement with BBP-II. Finally, the amino acid analyses of both BBP-I and BBP-II were practically identical. These sets of data could be rationalized if BBP-I were converted into BBP-II by limited proteolysis, a phenomenon suggested by Seshagiri & Adiga (1987).

Assays for the presence of biotin-binding proteins in the egg-whites and egg-yolks of some birds showed great species-variability in the amounts of avidin and yolk biotin-binding proteins (White, 1985; Korpela *et al.*, 1981). In the present paper we have also demonstrated biotin-binding activity among different species. Our results indicate chickens are the only species tested with distinct BBP-I and BBP-II activity. Furthermore, we have also demonstrated immunological similarities between chicken egg-yolk BBP-II and biotin-binding proteins from other species. BBP in turkey egg-yolk is distinctive in that it readily exchanges biotin at relatively low temperatures (White *et al.*, 1987) and yet is stable at 65 °C. In contrast, alligator egg-yolk BBP, which is also stable at 65 °C, does not readily exchange biotin at 40 °C (Table 3). The physiological significance of these exchange and stability properties is not clear; however, these properties may be useful in various applications now limited to avidin.

Note added in proof (received 24 October 1988)

In Table 2 histidine is identified as residue 5. Recovery of this amino acid was low. The homologous position in avidin is cysteine. Since BBP-II was not reduced and carboxymethylated before Edman analysis, cysteine, if present, would not have been detected.

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