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PARTITION OF PURIFIED HUMAN THYROXINE-BINDING GLOBULIN IN AQUEOUS TWO-PHASE SYSTEMS IN RESPONSE TO REACTIVE DYES

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SUMMARY

Thyroxine-binding globulin was isolated from pooled human serum by a two-step method involving affinity chromatography on thyroxine-Sepharose and preparative disc-electrophoresis. The final product was found to be homogeneous by polyacrylamide gel electrophoresis and has a molecular weight of 59 000. Isoelectric focusing resolved the protein into seven bands with isoelectric points ranging from 3.9 to 4.3.

The isolated protein showed affinity to a number of different dyes as recognized by affinity phase partitioning. The interaction of the protein with the dye Cibacron Blue F3G-A was found to be strongly competitive with the natural ligand thyroxine.

INTRODUCTION

Thyroid hormones in blood are bound to albumin, prealbumin and thyroxine-binding globulin. Although thyroxine-binding globulin is present only in tiny amounts it binds more than 70% of the total hormones in the blood with high affinity[1]. This protein was found to have a monomeric structure with a molecular weight of 58 000–65 000[2,3] and is able to bind one molecule of thyroxine per molecule of protein[1]. Effective purification methods for this protein have been elaborated by affinity chromatography applying immobilized thyroid hormones as ligands[2,4–6]. The main disadvantages of using natural ligands for affinity chromatography are their chemical instability and the high costs. These problems were found to be overcome for a number of proteins by using synthetic dyes that have binding properties similar to those of the natural ligands[7]. Successful application of dyes in the purification of serum proteins has already been practised in the cases of albumin, prealbumin and of other serum proteins (7–9).

Affinity partition has been used in the search for dye-binding sites in serum proteins[10,11]. The basis of the method is the partition of proteins in aqueous two-

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phase systems composed of poly(ethylene glycol) and dextran, and also the change of the partition coefficient when dye–polymer derivatives are included in the system.

The present paper describes the application of this method to the study of the dye-binding properties of thyroxine-binding globulin. Prior to this study it was necessary to isolate this protein from human serum. This was accomplished by a two-step procedure involving affinity chromatography on thyroxine-Sepharose and preparative disc-electrophoresis.

EXPERIMENTAL

Chemicals

Poly(ethylene glycol), M_r = 6000–7500 (PEG 6000), was from Ferak (Berlin, F.R.G.). Dextran T 500, M_r = 500 000, epoxy-activated Sepharose 6B and Cibacron Blue F3G-A were purchased from Pharmacia (Uppsala, Sweden). Remazol Yellow GGL was a gift from Hoechst (Frankfurt, F.R.G.). Procion dyes were products of ICI Organics Division (Blackley, U.K.). Agarose and anti-thyroxine-binding globulin serum from rabbits were from Behringwerke (Marburg, F.R.G). [^{125}I]thyroxine was obtained from Isocommerz (Berlin, G.D.R.). All other chemicals used were of analytical reagent grade. Ampholyte solutions and protein standard (protein mixture 9) for isoelectric focusing were from Serva (Heidelberg, F.R.G.).

Isolation of thyroxine-binding globulin

Thyroxine-binding globulin (TBG) was isolated from fresh pooled human serum by affinity chromatography using epoxy-activated Sepharose 6B for immobilization of thyroxine, as proposed by Kagedal and Källberg[5]. Briefly, 15 ml of settled thyroxine-Sepharose were stirred overnight with 800 ml of serum at 4°C. After an excess of serum had been removed through a Büchner funnel, the gel was washed exhaustively with 1.5 l of buffer (50 m*M* sodium phosphate, pH 7.4, 150 m*M* sodium chloride) and then packed into a column (30 × 1.5 cm I.D.). The column was eluted with 50 m*M* sodium carbonate buffer (pH 9.5) at a flow-rate of 30 ml. The eluate was collected in 6-ml fractions in tubes prefilled with 3 ml of 50 m*M* sodium phosphate buffer (pH 6.0) for neutralization. After concentration of the TBG-containing fractions by ultrafiltration the protein was subjected to preparative disc-electrophoresis in 7.5% polyacrylamide gels using the same buffer system as described for analytical disc-electrophoresis[12]. The protein zone capable of binding radioactive thyroxine as analysed by a reference gel was cut out of the gel and extracted several times with 50 m*M* sodium phosphate buffer (pH 7.4) containing 150 m*M* sodium chloride. Estimation of the molecular weight of TBG was performed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) according to Weber and Osborn[13].

Isoelectric focusing was performed as described in ref. 14. Phase partition experiments were carried out in 2-g systems prepared from stock solutions of dextran T 500 (20% w/w), PEG 6000 (40% w/w), buffer, protein and additives giving a composition as described in the respective figures. The dye-PEG derivatives were prepared according to Johansson *et al.*[10]. The partition coefficient, K, of TBG was determined as the ratio of the concentration in the top phase in the bottom phase. The term $\Delta \log K$ defines the difference in the partition of TBG in the presence and

in the absence of dye-PEG in the system. The concentration of the proteins in the two phases was determined immunologically by rocket electroimmunodiffusion[15].

RESULTS

Purification of TBG

Fig. 1 shows the electrophoretic analysis of the products obtained on different purification steps of TBG. After chromatography on immobilized thyroxine two dominating bands appeared. One of the bands was identified as TBG by its capability of binding radioactively labeled thyroid hormone. Prealbumin and albumin, the two other hormone-binding proteins, were present only in trace amounts in the preparation as immunologically analysed. Final purification of TBG was achieved by preparative disc-electrophoresis yielding a homogeneous protein with a recovery of *ca.* 30%. Thus, starting from 800 ml of serum containing 11 mg of TBG, 3 mg of pure protein were obtained.

The purified TBG migrates in the SDS electrophoresis as a single protein band with a molecular weight of 59 000. When TBG is subjected to isoelectric focusing it

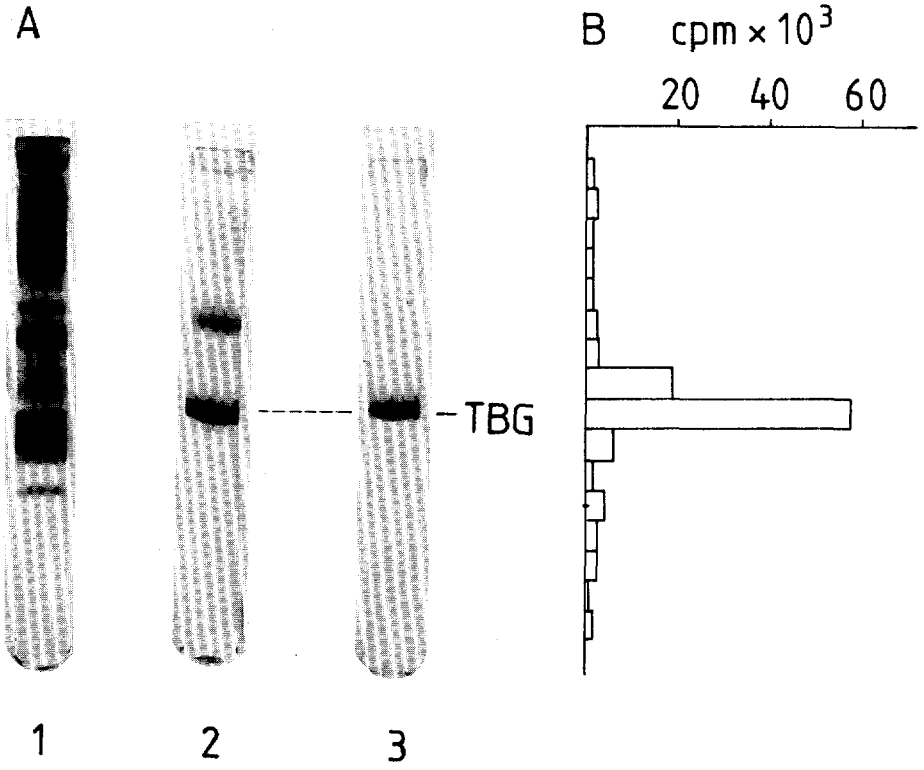


Fig. 1. Polyacrylamide gel electrophoresis of TBG at different stages of purification. (A) Disc-electrophoresis was performed in polyacrylamide gel gradients of 3–15% for 2 h and 3 mA per tube; 1 = human serum (400 μg); 2 = TBG after affinity chromatography (90 μg); 3 = TBG after preparative electrophoresis (30 μg). (B) Identification of TBG by labeling with radioactive [^{125}I]thyroxine; prior to electrophoresis the protein solution was incubated with [^{125}I]thyroxine for 30 min at room temperature. After electrophoresis the gel cylinder was cut into 0.5-cm slices and subjected to gamma counting.

shows a strong heterogeneity resolving into seven bands with isoelectric points in the range 3.9–4.3. The two main bands (arrows) exhibit isoelectric points of 4.0 and 4.1 (Fig. 2).

Partition experiments

Basic information on the partition behaviour of TBG in two-phase systems was obtained by studying the influence of parameters such as the pH value, buffer composition, ionic strength and concentration of the phase-forming polymers. Fig. 3 demonstrates the influence of increasing the concentration of dextran and PEG on the partition of TBG. In either case the protein partitions in favour of the dextran-rich phase with increasing polymer concentration. The effect of salts on partition is depicted in Fig. 4. The decreasing K value was found to be related to the concentration of the various salts rather than to the nature of the salts. The pH dependence of the partition of TBG is shown in Fig. 5, and was found to be markedly influenced by the buffer composition. In the presence of Tris–HCl or acetate buffer the partition of TBG becomes nearly independent of the pH in the respective range. In contrast, the partition coefficient of TBG increases strongly in the presence of phosphate buffer. By taking the different effects of these parameters into account, the partition of TBG can be directed to the lower phase by using phase systems composed of 8%

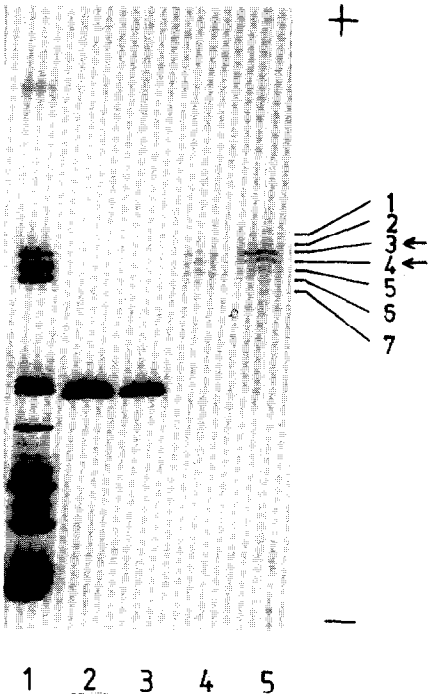


Fig. 2. Isoelectric focusing of TBG. Separation was performed in 5% polyacrylamide gel slabs (2 mm thickness) containing 8 M urea and 0.83% (v/v) Servalyt 2-11, 1.66% (v/v) Servalyt 3-5 and 0.83% (v/v) Servalyt 3-4. As reference the pI marker protein mixture 9 (Serva) was used. Anode solution: 1 M phosphoric acid; cathode solution: 1 M sodium hydroxide. 1 = Marker protein mixture; 2 and 3 = human serum albumin; 4 and 5 = purified TBG.

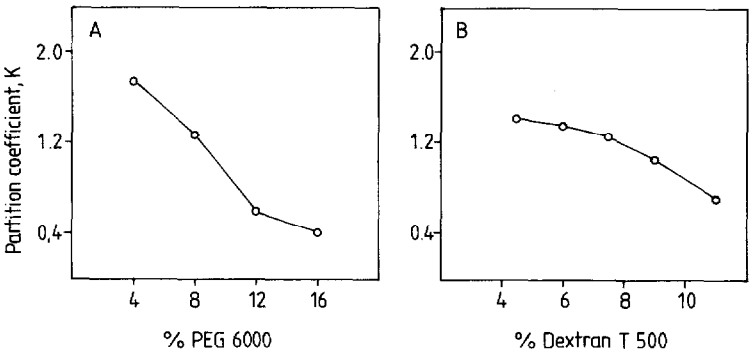


Fig. 3. Effect of the polymer concentration on partition of TBG. (A) Phase composition: 8% (w/w) dextran T 500, increasing concentration of PEG 6000 (w/w), 50 m*M* sodium phosphate buffer (pH 7.0), 80 μg of TBG per 2-g system. (B) Phase composition: 8% (w/w) PEG 6000, increasing concentration of dextran T 500 (w/w), 50 m*M* sodium phosphate buffer (pH 7.0), 80 μg of TBG per 2-g system. Temperature, 22°C.

dextran T 500, 8% PEG 6000 in 50 m*M* Tris–HCl (pH 7.0). The partition coefficient of TBG in such systems was found to be 0.22.

Affinity phase partitioning

When dye-liganded PEG derivatives are included in the system, the partition coefficient of TBG increases. Various of dyes exert different effects (Table I). It is obvious that TBG possesses affinity for all these dyes despite their great structural differences. Compared with human albumin, there are similarities in the relative affinity partitioning effect of the applied dyes. In contrast, prealbumin displays a low affinity for most of the dyes, except Remazol Yellow GGL.

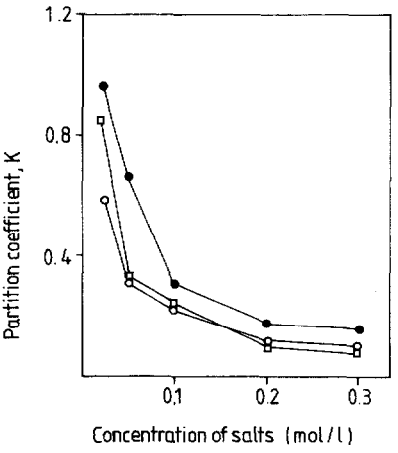
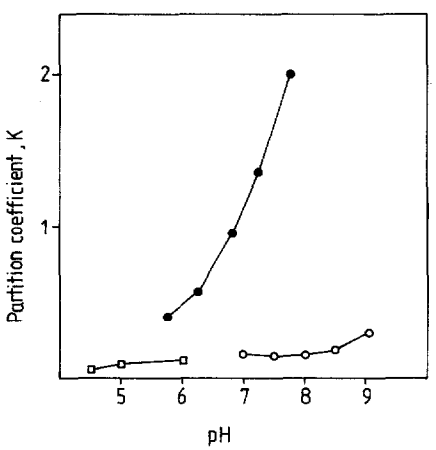


Fig. 4. Effect of salts on partition of TBG. Phase composition: 8% (w/w) PEG 6000, 8% (w/w) dextran T 500, 50 m*M* sodium phosphate buffer (pH 7.0), and increasing concentration of salts, 80 μg of TBG per 2-g system. Temperature, 22°C. Symbols: ○ = sodium isothiocyanate; ● = sodium chloride; □ = sodium iodide.



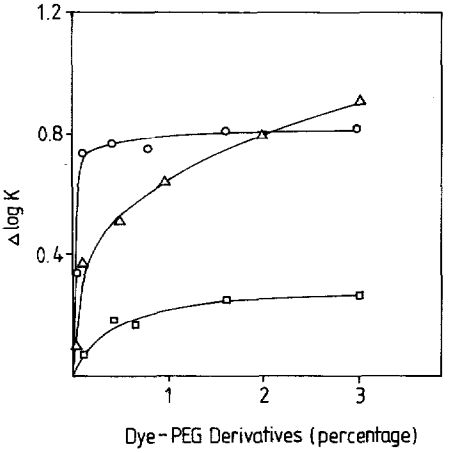


Fig. 5. Dependence of partition of TBG on the pH and buffer composition. Phase composition: 8% (w/w) PEG 6000, 8% (w/w) dextran T 500, 80 μg of TBG and buffers of different pH values. Symbols: ● = 50 m*M* sodium phosphate buffer; ○ = 50 m*M* Tris–HCl buffer; □ = 50 m*M* sodium acetate buffer. Temperature, 22°C.

Fig. 6. Partition of TBG as a function of the concentration of PEG-bound dyes. Phase composition: 8% (w/w) total PEG including dye–PEG derivatives of increasing concentration, 8% (w/w) dextran T 500, 50 m*M* Tris–HCl (pH 7.0), 80 μg of TBG per 2-g system. Symbols: ○ = Procion Green H-4G-PEG; △ = Cibacron Blue F3G-A-PEG; □ = Remazol Yellow GGL-PEG.

TABLE I

EFFECT OF DYE–PEG DERIVATIVES ON PARTITION OF TBG IN COMPARISON WITH ALBUMIN (HSA) AND PREALBUMIN (PA)

Phase composition for TBG: 8% (w/w) total PEG 6000 including 2% dye–PEG, 8% (w/w) dextran T 500, 50 m*M* Tris–HCl (pH 7.0), 80 μg of TBG per 2-g system. Phase composition for HSA and PA: 7.5% (w/w) total PEG including 2% dye–PEG, 10% (w/w) dextran T 500, 20 m*M* sodium phosphate buffer (pH 7.0), 130 μg of prealbumin and 2 mg of albumin per 2-g system. The log *K* values of PA and HSA in the absence of dye–PEG derivatives were −0.38 and −1.37, respectively.

Dye–PEG derivatives	*Δlog K*		
	TBG	*HSA*	*PA*
Procion Red HE-3B	0.64	0.98	0.04
Procion Yellow HE-4R	0.40	0.50	0.05
Procion Navy HE-R	0.76	1.2	0.10
Procion Brown H-EG	0.70	1.2	0.11
Procion Blue MX-3G	0.63	1.76	0
Procion Blue MX-R	0.25	0.82	0.16
Procion Yellow MX-R	0.21	1.00	0.12
Procion Orange MX-G	0.23	0.92	0
Procion Green H-4G	0.83	1.36	0.02
Procion Red HE-7B	0.45	0.88	0.02
Procion Red H-3B	0.22	0.70	0
Procion Brown MX-5BR	0.81	1.38	0
Cibacron Blue F3G-A	0.84	2.50	0
Remazol Yellow GGL	0.25	0.50	1.10

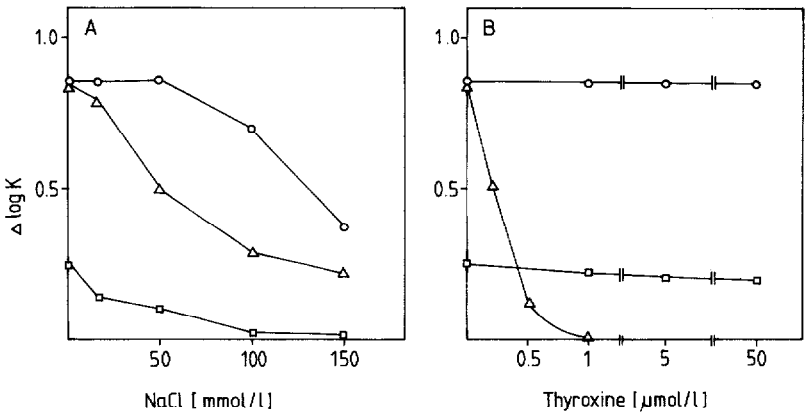


Fig. 7. Effect of sodium chloride and thyroxine on affinity partition of TBG. (A) Phase composition: 8% (w/w) PEG 6000 including 2% of dye–PEG, 8% (w/w) dextran T 500, 50 m*M* Tris–HCl (pH 7.0) and increasing concentration of sodium chloride, 80 μg of TBG per 2-g system. (B) Phase composition: 8% (w/w) PEG 6000 including 2% of dye–PEG, 8% (w/w) dextran T 500, 50 m*M* Tris–HCl (pH 7.0), 80 μg of TBG, 1.8 mg of albumin per 2-g system, and increasing concentration of thyroxine. Temperature, 22°C. Symbols (A and B): ○ = Procion Green H-4G-PEG; △ = Cibacron Blue F3G-A-PEG; □ = Remazol Yellow GGL-PEG.

Fig. 6 shows how the partition coefficient of TBG varies with the concentration of selected dye–PEG derivatives. In the presence of Cibacron Blue F3G-A or Procion Green H-4G the $\Delta\log K$ value of TBG increases markedly as the dye–PEG concentration increases. Since the steepness of the curves is related to the binding constant of the dye–protein complex, a higher affinity of TBG for Procion Green H-4G than for Cibacron Blue F3G-A may be deduced. The influence of Remazol Yellow GGL-PEG on the partition of TBG was found to be rather low. A maximum $\Delta\log K$ value of 0.25 units was found.

The binding of TBG to the dyes can be reduced by increasing the salt concentration (Fig. 7A). Even at a concentration of 150 m*M* sodium chloride the binding to Cibracon Blue F3G-A and to Procion Green H-4G is markedly diminished, and it has completely disappeared in the case of Remazol Yellow GGL.

Dyes are known to bind either specifically close to the sites of the natural ligands or more unspecifically to other parts of the protein surface. These interactions can be distinguished by measuring the effect of natural ligands on the dye–protein interaction. Thus, in the case of TBG the competing action of thyroxine was studied for its ability to decrease the $\Delta\log K$ value for the respective dyes. As demonstrated in Fig. 7B, thyroxine up to a concentration of 50 μ*M* was found to have no significant effect on the binding of TBG to Procion Green H-4G or to Remazol Yellow GGL. However, a very strong competition was evident between thyroxine and Cibacron Blue F3G-A. TBG does not bind to this dye even at thyroxine concentrations in the nanomolar range.

DISCUSSION

Efficient purification methods for TBG have been described[2,4–6], all of which

combine affinity chromatography as most effective procedure with at least two other conventional chromatographic steps, *e.g.* gel filtration, ion-exchange chromatography. We intentionally excluded the application of ion-exchange chromatography in order to preserve the original microheterogeneity of TBG reported by Horn *et al.*[16]. Our product was found to consist of at least seven different protein components. Protein heterogeneity is often determined genetically as it is known from proteinase inhibitor[17]. It would be particularly interesting to know the reason for polymorphism in the case of TBG. Since TBG is the most important thyroxine acceptor in the blood, this knowledge would presumably permit a deeper understanding of the normal and pathological states of the thyroid hormones.

Interestingly, serum proteins with a specific transport function, such as albumin, prealbumin and vitamin D-binding protein, show rather specific binding properties towards synthetic dyes[18–20]. Other serum proteins also bind to the dyes but in a more or less unspecific manner[21]. Reactive dyes have been applied in biochemistry not only for purposes of affinity chromatography but also as useful probes for targetting of active sites in proteins[22]. To explore such dye-binding sites in TBG we employed the method of affinity partitioning in two-phase systems. In the absence of dye–PEG the partitioning of TBG followed the general behaviour of other proteins and could be influenced by a variety of parameters[23–25]. The study of these parameters is necessary in order to know how to direct the partitioning of a protein into one phase, which is the best way of measuring the effect of dye-substituted polymers.

Like albumin, TBG showed an unexpectedly strong binding to a great variety of dyes. This property is not surprising in albumin, because it has a number of different binding sites for fatty acids, bilirubin, thyroxine, tryptophan and a great diversity of drugs. Since TBG possesses similar dye-binding properties one should expect TBG to have binding sites in addition to sites of thyroxine binding. It is thus interesting to note that TBG also has hydrophobic sites for fatty acid binding[2].

In general, complexation of dyes with proteins occurs at apolar regions of the protein surface that are surrounded by ionic groups. Since dyes as well as natural ligands possess completementary structures, they often share common binding sites in proteins. In this sense, the binding of TBG to Cibacron Blue F3G-A can be regarded as a rather specific interaction because of the strong competing effect to thyroxine. It is therefore important to recognize specific dye–protein interactions in order to use dyes for separation and purification of proteins.

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