Solubilization and purification of a membrane-associated 3,3',5-tri-iodo-L-thyronine-binding protein from rat erythrocytes

Roberto C. ANGEL, Joaquín A. BOTTA, Roberto D. MORERO* and Ricardo N. FARIAS

Instituto de Química Biológica 'Dr. Bernabé Bloj', Facultad de Bioquímica, Química y Farmacia, Departamento de Bioquímica de la Nutrición, INSIBIO/UNT/CONICET, Chacabuco 461, 4000 San Miguel de Tucumán, Argentina

3,3',5-Tri-iodo-L-thyronine (L-T₃) binding sites from rat erythrocyte membranes were solubilized in an active form by using the zwitterionic detergent CHAPS or the anionic detergent lauroylsarcosine. The binding protein was successively purified by Sephadex G-200 and affinity chromatography. The purified material retained its binding activity and exhibited high affinity and specificity compared with those displayed in the original membrane. Yield was about 10% of the starting activity. The specific binding activity was enriched by approx. 100-fold, which represents a purity of only 0.1%. Analysis of the purified preparation on SDS/PAGE showed two major protein bands (*M*_r 64 000 and *M*_r 50 000), but these could not represent the binding protein since the purity obtained was low. However, affinity-labelling experiments with *N*-bromoacetyl-L-[¹²⁵I]T₃ in intact membranes showed that two proteins (also with *M*_r values of 64 000 and 50 000) bound the hormone specifically, suggesting a co-migration of hormone receptors and contaminants on gel electrophoresis.

INTRODUCTION

It is believed that the primary physiological effect of thyroid hormone on target cells is exerted through its binding to nuclear sites. The major results of this interaction are the stimulation of the basal metabolic rate [1–7] and the regulation of tissue differentiation and development [8]. However, numerous reports have also described 3,3',5-tri-iodo-L-thyronine (L-T₃) binding sites in other subcellular fractions such as mitochondria [9], cytosol [10] and plasma membrane from several tissues, including placenta [11], liver [12], pituitary tumour GH₃ [13], thymocytes [14] and erythrocytes [15,16]. The physiological role of these extranuclear binding sites remains unclear. It is postulated that binding of L-T₃ to the plasma membrane has at least three effects: (1) it initiates the uptake of the hormone by the cell [17–19]; (2) it increases amino acid and sugar transport into the cell [20,21]; and (3) it changes the membrane fluidity and the kinetic behaviour of several membrane-bound enzymes [22].

In previous papers [15,16] we have described the presence of high-affinity L-T₃ binding sites in rat and human erythrocyte membranes which have been found to be integral and peripheral proteins respectively. Hitherto, plasma membrane L-T₃ binding proteins have only been solubilized from human placenta [11], rat pituitary tumour GH₃ [23] and human erythrocytes [16]. Further characterization of the L-T₃ receptor can be achieved by solubilization and purification of the receptor in its active form.

In this report we describe the solubilization of L-T₃ binding sites from rat erythrocyte membranes and some of their properties, as well as their purification by using Sephadex G-200 and affinity chromatography.

MATERIALS AND METHODS

Materials

L-[¹²⁵I]T₃ (specific radioactivity 2200 µCi/µg) was obtained from New England Nuclear. Lauroylsarcosine, SDS, sodium deoxycholate, Brij 58, Triton X-100, Tween 40, CHAPS, octyl

glucoside, phenylmethanesulphonyl fluoride (PMSF), leupeptin, Sephadex, Sepharose and Dowex were from Sigma. Octaethylene glycol dodecyl ether (OEDGE) was purchased from Nikko Chemicals, Tokyo, Japan. All other chemicals were of reagent grade and were used without further purification.

Membrane preparation

Male Sprague–Dawley rats weighing 200–300 g, fed on a standard diet, were used to obtain erythrocyte membranes. Blood was drawn by heart puncture. Erythrocytes were separated by centrifugation and washed as previously described [15], and erythrocyte ghosts were prepared by the method of Dodge *et al.* [24] ('Dodge membranes'). Peripheral proteins were released by washing three times with 10 vol. of 2 mM-EDTA, pH 11.0 (adjusted with 0.5 M-NaOH) [25] ('alkali-treated membranes'). After each treatment the membranes were collected by centrifugation at 40 000 *g* for 15 min at 4 °C and finally resuspended in phosphate-buffered saline (PBS) (138 mM-NaCl, 10 mM-sodium phosphate, pH 7.4) and stored at 4 °C.

Detergent solubilization of membrane L-T₃ binding sites

Solubilization of L-T₃ binding sites was accomplished by suspending the membranes (2–4 mg of protein/ml) in PBS containing PMSF (0.5 mM), leupeptin (1 µg/ml) and EDTA (1 mM), in the presence of different detergent concentrations. The suspension was incubated on ice for 15 min and the particulate material was then removed by centrifugation at 100 000 *g* for 30 min. The supernatant was concentrated on a P-10 Amicon membrane and stored at 4 °C until used (no more than 1 day).

Sephadex G-200 chromatography

Concentrated supernatants (2 mg of protein/ml) from detergent treatment with 10 mM-lauroylsarcosine or 5 mM-CHAPS were incubated with 0.1 nM-L-[¹²⁵I]T₃ for 45 min at 4 °C. The mixture was applied to a 1.0 cm × 55 cm Sephadex G-200 column pre-equilibrated with 0.1% lauroylsarcosine/20 mM-phosphate

Abbreviations used: L-T₃, 3,3',5-tri-iodo-L-thyronine; OEDGE, octaethylene glycol dodecyl ether; PBS, phosphate-buffered saline; D-T₃, 3,5,3'-tri-iodo-D-thyronine; L-T₄, L-thyroxine; TRIAC, 3,5,3'-tri-iodothyroacetic acid; CMC, critical micelle concentration; BrAc-L-[¹²⁵I]T₃, *N*-bromoacetyl-3,3',5-tri-iodo-L-thyronine; PMSF, phenylmethanesulphonyl fluoride.

* To whom correspondence should be addressed.

Dedicated to Raúl E. Trucco on the occasion of his 75th birthday.

buffer, pH 7.6. Elution was carried out with the same buffer. Fractions of 1.0 ml were collected at 4 °C and monitored for protein (A_{280}) and for radioactivity. Fractions with L-T₃ binding activity were pooled.

Affinity chromatography

L-T₃-CH Sepharose 4B was prepared by incubating swollen washed CH-Sepharose 4B with L-T₃ dissolved in water/dioxan (1:1, v/v), pH 5.0–6.0, and 0.1 M-*N*-ethyl-*N'*-(3-dimethylamino-propyl)carbodi-imide hydrochloride for 24 h at room temperature with gentle stirring. The gel was then consecutively washed with water/dioxan (1:1, v/v), ethanolamine (pH 8.3), 0.1 M-Tris/HCl buffer (pH 8.3)/0.5 M-NaCl, 0.1 M-sodium acetate buffer (pH 4.0)/0.5 M-NaCl and PBS.

L-T₃ binding sites solubilized from alkali-treated membranes or eluted from the Sephadex G-200 column were bound to the affinity gel by batchwise adsorption. In most experiments, a 1–5 ml sample (1–10 mg of protein) was applied per ml of bed volume. After incubation for 4 h at 4 °C, with occasional mixing, the gel was washed five times with 10 vol. of PBS, pH 7.6. Batchwise elution of the L-T₃ binding sites was performed by incubating the gel with PBS containing 1 μ M-L-T₃ and 0.05 % lauroylsarcosine or 0.025 % CHAPS for 2 h at 4 °C. After centrifugation at 800 *g* for 5 min, the supernatant was extensively dialysed against PBS to remove free L-T₃. Control experiments indicated that equivalent amounts of L-[¹²⁵I]T₃ added to the mixture were removed completely after 12 h of dialysis (results not shown).

SDS/polyacrylamide gel electrophoresis

One-dimensional gel electrophoresis was performed in 1 mm-thick SDS/10 % polyacrylamide gels according to Laemmli [26]. Samples (80 μ l) from each purification step were electrophoresed for 12 h at a constant current of 10 mA. The gel was stained with Coomassie Blue, destained and dried.

L-[¹²⁵I]T₃ binding assay

Samples (50 μ l) of membrane suspension soluble material (50–200 μ g of protein) or detergent solution were incubated with 50 pM-L-[¹²⁵I]T₃ (approx. 50000 c.p.m.) for 30–60 min at 4 °C in 0.5 ml of PBS. Specific binding was determined by the addition of an excess of unlabelled L-T₃ (0.1 μ M). After incubation, bound and free ligand were separated by a Dowex exchange resin method according to Bernal *et al.* [27]. Briefly, 50 μ l of a Dowex 1X8 (Cl⁻ form; 200–400 mesh) suspension (600 mg/ml) was added to the reaction mixture. The tubes were immediately shaken for 2 min and centrifuged at 1000 *g* for 1 min; 0.3 ml portions of the supernatant were counted for radioactivity in an Espectromatic Counter. Specific L-T₃ binding was calculated by subtracting non-specific L-T₃ binding from total L-T₃ binding.

Affinity labelling of L-T₃ binding protein with BrAc-L-[¹²⁵I]T₃

BrAc-L-[¹²⁵I]T₃ was synthesized as described [13]. 'Dodge membranes' (100 μ g of protein) were incubated with 0.5 nM-BrAc-L-[¹²⁵I]T₃ in PBS at 4 °C for 4 h. After the incubation, the membranes were washed twice with PBS to remove the labelling reagent which was not covalently bound and its hydrolysis products. Samples were electrophoresed as indicated above and the dried gel was autoradiographed for 96 h at –60 °C using a Kodak AR film.

Other methods

Protein content was estimated by the method of Bradford [28] or by the fluorescamine assay [29]. The critical micelle concentration (CMC) was measured using the fluorescence en-

hancement of anilidonaphthalenesulphonate, excited at 360 nm, which accompanies its association with lauroylsarcosine micelles.

RESULTS

Solubilization of membrane L-T₃ binding sites

One of the fundamental approaches in understanding the biochemistry of membrane receptors is their solubilization in an active form. The L-T₃ binding site from rat erythrocyte membranes behaves like an integral protein [15] and therefore its solubilization requires detergent treatment. In the choice of detergent, three factors of practical importance were taken into account. First, detergent monomers or micelles had to interact only minimally with L-[¹²⁵I]T₃. This could enable us to more accurately evaluate specific L-[¹²⁵I]T₃ binding to soluble sites even in the presence of detergent. Second, the detergent had to efficiently extract the binding sites from the membrane. Third, the sites had to be solubilized without loss of binding activity. Determination of L-T₃ binding to ionic and non-ionic detergents at different concentrations is shown in Fig. 1. As can be seen, the non-ionic detergents OEGDE, Brij 58, Triton X-100 and Tween 40 all bound a high percentage of total radioactivity at concentrations greater than 0.1 %. These concentrations clearly exceeded the CMC. Since these detergents at concentrations lower than 0.1 % were unable to solubilize any appreciable amount of membrane protein, we decided against their use for the solubilization of the L-T₃ binding protein.

The anionic (lauroylsarcosine, sodium deoxycholate, SDS) as well as the non-ionic (octyl glucoside) and zwitterionic (CHAPS) detergents did not bind L-T₃ significantly at detergent concentrations up to 0.3 %. Sodium deoxycholate, SDS and octyl glucoside were able to solubilize large amounts of membrane protein, but the L-T₃ binding sites were totally inactivated or blocked on solubilization (results not shown). After this trial-and-error search for the optimal detergent, only CHAPS and lauroylsarcosine met the above requirements: i.e. they did not bind L-T₃ non-specifically at concentrations lower than the CMC (Fig. 1), and they efficiently solubilized L-T₃ binding sites in an active form (Table 1). From the range of concentrations tested, it is concluded that 0.24 % (5 mM) CHAPS and 0.3 % (10 mM)

Fig. 1. L-[¹²⁵I]T₃ binding to detergents

Different concentration of OEGDE (●), Brij 58 (○), Triton X-100 (■), Tween 40 (□), lauroylsarcosine (△), deoxycholate (▲), SDS (■), CHAPS (▲), and octyl glucoside (△) [with CMC values of 0.025, 0.01, 0.02, 0.006, 0.28, 0.15, 0.20, 0.45 and 0.73 % (w/v) respectively] were incubated with L-[¹²⁵I]T₃ (0.1 nM). After 60 min at 4 °C, L-T₃ binding was measured as described in the text.

Table 1. Solubilization of L-T₃ binding sites from rat erythrocyte membranes

Solubilized protein and specific binding were measured in the supernatant after centrifugation of 100 000 *g* for 1 h.

Detergent		Solubilized protein (%)	Specific binding (fmol/mg of protein)
CHAPS	0.06 %	2	61
	0.12 %	6	62
	0.18 %	8	123
	0.24 %	11	180
	0.36 %	16	120
	0.48 %	22	40
Lauroylsarcosine	0.10 %	6	25
	0.20 %	22	38
	0.30 %	46	48
	0.40 %	62	34
	0.50 %	75	16
Membrane preparation (alkali-treated)		—	61

lauroylsarcosine gave the highest proportion of specific binding. These concentrations were used for solubilization and subsequent characterization studies.

Sephadex G-200 chromatography

L-T₃ binding sites from rat erythrocyte membranes were solubilized with 5 mM-CHAPS or 10 mM-lauroylsarcosine. After centrifugation at 100 000 *g* for 30 min the clear supernatant was incubated with 0.7 nM-L-[¹²⁵I]T₃ and fractionated in a Sephadex G-200 column. As shown in Fig. 2, two binding peaks were detected. A minor peak was excluded from the gel and a major peak co-eluted with bovine serum albumin standard, at an estimated *M_r* of 64 000. When an excess (0.1 μM) of unlabelled L-T₃ was added to the supernatant, only the *M_r*-64 000 peak was affected, decreasing by about 30 %.

Affinity chromatography

L-T₃ binding sites solubilized from membranes, or the pooled binding fractions from the Sephadex G-200 column, were mixed with an affinity matrix containing immobilized hormone. Non-specifically bound proteins were removed by washing, and specifically bound L-T₃ binding sites were eluted by adding an excess of unlabelled hormone, as described in the Materials and methods section. The eluate was dialysed to eliminate unlabelled

Fig. 2. Sephadex G-200 chromatography of solubilized L-T₃ binding proteins

The CHAPS extract from alkali-treated membranes (2 mg/ml) was incubated for 1 h at 4 °C with 0.1 nM-L-[¹²⁵I]T₃ in the absence or presence of 0.1 μM unlabelled L-T₃ and applied to a Sephadex G-200 column equilibrated with PBS containing 20 mM-sodium phosphate (pH 7.6)/0.1 % lauroylsarcosine. The column was eluted with the same buffer. The fractions (1.0 ml each) were monitored for absorbance at 280 nm (—), total L-[¹²⁵I]T₃ binding activity (●) and non-specific L-[¹²⁵I]T₃ binding activity (○). The arrows indicate the void volume (*V*₀) and the standard proteins of known *M_r*: BSA (67 000) and myoglobin (Myo; 16 950).

L-T₃, incubated with L-[¹²⁵I]T₃ and finally subjected to gel filtration through a Sephadex G-200 column equilibrated and eluted as described before. The binding activity eluted with a profile similar to that shown in Fig. 2, except for the absence of the minor peak (results not shown).

Purification achieved

L-T₃ binding sites were enriched by approx. 100-fold after the affinity chromatography step. Table 2 summarizes the overall purification procedure. As can be seen in the electrophoretogram in Fig. 3(a), which shows samples containing L-T₃ binding activity at various stages of purification, the key step was the affinity chromatography. The yield obtained was low (10 %), and the preparation showed only two major bands, of *M_r* 64 000 (p64) and *M_r* 50 000 (p50), as characterized by SDS/PAGE (Fig. 3a, lane 4). Even so, the achieved purity was very low (0.1 %) and the implication of this will be discussed below.

Table 2. Erythrocyte L-T₃ binding protein purification

Preparation	Total protein (mg)	Total binding (fmol of L-T ₃)	Specific binding (fmol of L-T ₃ /mg of protein)	Purification (fold)	Yield (%)
'Dodge membrane'	29.6	1036	35	—	100
Alkali-treated membrane	12.0	732	61	1.7	71
Detergent-solubilized membrane	1.56	353	180	5.1	34
Sephadex G-200	0.55	215	390	11.2	21
Affinity chromatography	0.03*	104	3480	100	10

* Value determined by fluorescamine assay.

Fig. 3. SDS/PAGE of L-T₃ binding sites

(a) Electrophoretic patterns at various stages of L-T₃ binding site purification: 'Dodge membranes' (lane 1), alkali-treated membranes (lane 2), CHAPS extract (lane 3) and material purified by affinity chromatography (lane 4). Proteins were stained with Coomassie Blue. (b) Autoradiogram of BrAc-L-[¹²⁵I]T₃-labelled proteins. 'Dodge membranes' (100 µg of protein) were affinity-labelled with BrAc-L-[¹²⁵I]T₃ (for further details, see the Materials and methods section).

Fig. 4. Ability of different L-T₃ analogues to displace L-[¹²⁵I]T₃ from partially purified L-T₃ binding sites eluted from Sephadex G-200

L-T₃ binding sites were incubated with 0.1 nM-L-[¹²⁵I]T₃ in the presence of various concentrations of TRIAC (■), D-T₃ (▲), L-T₄ (●), and L-T₃ (○). Bound L-[¹²⁵I]T₃ was determined and expressed as a percentage of L-[¹²⁵I]T₃ bound in the absence of unlabelled analogue.

It was observed that the first and second steps of the purification procedure (alkaline wash of 'Dodge' membranes and Sephadex G-200 chromatography) could be omitted, since when the Dodge membranes were directly solubilized and the extract subjected to affinity chromatography, similar results were obtained (results not shown).

Binding characteristics of the material partially purified by Sephadex G-200

Fig. 4 shows a study of the binding affinities of several thyroid hormone analogues relative to that of L-T₃. In these experiments, the ability of unlabelled L-T₃, L-thyroxine (L-T₄), 3,5,3'-tri-iodo-D-thyronine (D-T₃) and 3,5,3'-tri-iodothyroacetic acid (TRIAC) to compete with L-[¹²⁵I]T₃ for the L-T₃ binding sites eluted from the Sephadex G-200 column was evaluated. L-T₃ was the most effective in displacing L-[¹²⁵I]T₃ from its binding sites. The rank-order relative binding affinities of the different analogues were: L-T₃ > L-T₄ > D-T₃ > TRIAC. When compared with L-T₃, about a

Fig. 5. Scatchard analysis of L-[¹²⁵I]T₃ binding to partially purified L-T₃ binding sites

The pool of active fractions eluted from the Sephadex G-200 column was assayed for hormone binding. Protein (25 µg) was incubated with various concentrations of L-[¹²⁵I]T₃ (from 5–300 pM). To determine the specific binding, the non-specific binding (determined in the presence of 0.1 µM unlabelled hormone) was subtracted from total binding.

1000-fold higher concentration of L-T₄ was necessary to displace 50% of the specifically bound L-[¹²⁵I]T₃. This binding affinity parallels that of the original L-T₃ binding sites in starting membrane preparations [15].

Saturation studies of L-T₃ binding according to Scatchard [30] (Fig. 5) revealed the presence of two sets of saturable binding sites, one of higher affinity and lower capacity (K_d 36 ± 4 pM; B_{max} 70 ± 19 fmol/mg of protein) and the other of lower affinity and higher capacity (K_d 220 ± 12 pM; B_{max} 190 ± 90 fmol/mg of protein). Values are means ± S.D. of four separate preparations. Their binding characteristics are very similar to those described for the starting material (alkali-treated membranes).

Affinity labelling with BrAc-L-[¹²⁵I]T₃

When erythrocyte membranes were affinity-labelled with BrAc-L-[¹²⁵I]T₃ (see the Materials and methods section), two proteins became labelled, as established by SDS/PAGE and subsequent autoradiography. The estimated M_r values of these proteins were 64 000 (p64) and 50 000 (p50) (Fig. 3b). A similar electrophoretic profile was obtained with the material purified by affinity chromatography and stained with Coomassie Blue R-250 (see Fig. 3a, lane 4).

DISCUSSION

A considerable body of evidence indicates that the effects of thyroid hormone are mediated by its interaction with a chromatin-associated receptor [31,32]. Although it was previously believed that L-T₃ enters the cell via passive diffusion [33], recent experiments with different cell types [11–16] have demonstrated the presence of high-affinity L-T₃ binding sites on the plasma membrane that could mediate hormone uptake.

Using a technique of affinity labelling, Horiuchi *et al.* [13] were able to detect a protein with an apparent M_r of 55 000 in cell extracts and highly purified plasma membranes from rat GH₃ cells. Simultaneously, Cheng *et al.* [34] purified and characterized a membrane-associated L-T₃ binding protein from A431 human epidermoid carcinoma cells which was detected on the nuclear envelope and endoplasmic reticulum by an indirect immunofluorescence technique. Later, Kitagawa *et al.* [35] isolated

another L-T₃ binding protein from A431 cells with an M_r of 58000 which was apparently different from that described by Cheng *et al.* [34]. On the other hand, a rat liver nuclear thyroid hormone receptor was characterized by Ichikawa & De Groot [36] as a 49000- M_r protein.

A comparative study of thyroid hormone receptors and binding proteins from nuclear, plasma membrane and cytosolic fractions of rat liver by photoaffinity labelling with L-T₄ was carried out by Dozin *et al.* [37]. However, the relationship of plasma-membrane-associated L-T₃ binding sites to nuclear receptors remains to be established. To elucidate the role of the specific membrane L-T₃ binding proteins in hormone action, a more detailed knowledge of these sites is required. In the present paper we provide a useful methodological approach for the isolation of L-T₃ binding sites from rat erythrocyte membranes [15]. We have successfully solubilized these receptors by using either the zwitterionic detergent CHAPS or the anionic detergent lauroylsarcosine, with preservation of both binding affinity and specificity.

When the crude CHAPS extract was fractionated by Sephadex G-200 chromatography, one major L-T₃ binding activity peak with an apparent M_r of 64000 was detected. The analysis of L-T₃ binding to the partially purified material demonstrated high- and low-affinity binding sites with K_d values of 30 pM and 220 pM respectively. These values are very close to those found in alkali-washed membranes [15]. Moreover, the L-T₃ binding sites exhibited high specificity for L-T₃, since TRIAC, D-T₃ and L-T₄ were much less effective than unlabelled L-T₃ in displacing L-[¹²⁵I]T₃.

After the affinity chromatography step, the purified protein retained L-T₃ binding activity, and compared with the total cellular extract the specific binding activity was enriched by nearly 100-fold. The activity of the purified material is very labile and therefore this could well represent a minimal estimate of the enrichment, as the binding activity could have experienced a gradual decay during the course of purification. In fact, storage of the purified material for 12 h at 4 °C resulted in a substantial decrease or complete loss of the initial activity (results not shown). One possible explanation for the observed decrease in activity is that the native conformation of the purified protein is not retained intact if associated with detergents. It seems likely that L-T₃ binding proteins have functionally important interactions with the membrane lipid environment and that specific requirements for lipid head-groups or intact phospholipid molecules have to be met for its biological activity to be expressed. Analysis of the purified material by SDS/PAGE gave two bands (M_r 64000 and 50000). These proteins could not be the hormone receptor, since 3500 fmol of binding protein (M_r 70000) with specific activity of 3500 fmol/mg of protein corresponded to 245 ng (assuming one binding site per molecule). Supposing that all the receptor molecules are active, 245 ng of binding protein/mg of protein represents a purity of less than 0.1%. However, 'Dodge membranes' labelled by incubation with BrAc-L-[¹²⁵I]T₃ and subjected to SDS/PAGE and subsequent autoradiography showed two radiolabelled products of the same M_r values as these obtained from the affinity chromatography step. These results suggest that the hormone receptor co-migrates with the major contaminants on gel electrophoresis.

Specific L-T₃ binding sites have been described in the nuclei of mammalian erythroid cells [38], nucleated erythrocytes of tadpole [39,40] and fish [41], and cytoplasm of mammalian erythrocytes [10,42]. On the other hand, binding of thyroid hormone to the surface of mammalian erythrocytes has been reported by several investigators [43–47]. With regard to the binding sites that we have isolated, it is pertinent to inquire into their physiological significance in the mature erythrocytes, as these cells are devoid of the subcellular organelles which are characteristically present

in thyroid-hormone-responsive tissues. They might well be viewed as vestigial structures, being functional only during the early development of the erythrocyte. It has been reported that L-T₃ stimulates erythropoiesis in human bone marrow cells *in vitro* [48]. On the other hand, we cannot exclude the possibility that the receptors play a role in the mature erythrocyte, and further studies are necessary to resolve these important questions.

The purified sites should allow the possibility of preparing specific antibodies and to study structure–function relationships. Moreover, it is of interest to explore the existence of these binding proteins in nucleated erythroid cells, their appearance in the course of the erythrocyte ontogenesis and how they relate to the other subcellular binding sites. These studies may well be assisted by the availability of specific antiserum.

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