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Note

Interaction of heme proteins and thyroid hormone

II. Localization of the site on thyroid hormone that binds to hemoglobin

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The binding of iodothyronines by hemoglobin[1] and myoglobin[2] has recently been described by this laboratory. Interaction of thyroid hormone and these heme proteins is poorly dissociable[1–3], suggestive of the apparently covalent complexes of iodothyronine and tissue proteins described by Surks and Oppenheimer[4]. The site or sites on thyroxine (T_4) and triiodothyronine (T_3) involved in binding to heme proteins have not been previously identified and are the subject of this report.

MATERIALS AND METHODS

Isotopic hormones

[^{125}I]T_4 and [^{125}I]T_3 were obtained from Amersham (Arlington Park, IL, U.S.A.) and their purity verified by thin-layer chromatography (TLC)[5]. [^{125}I]Tetraiodothyroacetic acid (TETRAC) was custom-synthesized from unlabeled triiodothyroacetic acid (Sigma, St. Louis, MO, U.S.A.) by New England Nuclear Corporation (Boston, MA, U.S.A.) and was re-chromatographed to purity in our laboratory. Labeled 3,3′,5′-triiodothyronine (reverse T_3, rT_3) was obtained from Serono (Braintree, MA, U.S.A.) as [^{125}I]rT_3 and was shown by TLC to contain a small amount of contaminating radioiodide. The rT_3 was used in binding studies without further purification.

Reagents

Purified human hemoglobin was purchased from Sigma; polyacrylamide gel electrophoresis at pH 9.0 (ref. 6) showed this preparation to be homogeneous.

Gel filtration

Sephadex G-100 (Pharmacia, Piscataway, N.J., U.S.A.) chromatography of solutions of hemoglobin and labeled iodothyronines was carried out as previously described[1], using 0.02 *M* phosphate buffer, pH 7.6 as the eluting buffer. Fractions were monitored for absorbance at 555 nm (deoxyhemoglobin) and radioactivity.

Bound/free partition of labeled T_4 and T_3 in solutions of hemoglobin

In order to determine the possible effects of phenolic hydroxyl group ionization

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of iodothyronines on the binding of the latter to hemoglobin, we measured hemoglobin binding of [^{125}I]T_3 and [^{125}I]T_4 in phosphate-buffered solutions of hemoglobin at various pH values. Bound/free partition of hormones was quantitated by the addition of dextran-coated charcoal[1]. "Control binding" at each pH was defined as the amount of hormone remaining in the supernatant of buffer without hemoglobin after the addition of charcoal and was subtracted from the bound hormone which was measured in the presence of hemoglobin.

RESULTS

Gel filtration of purified hemoglobin previously reacted with radioactive TETRAC, rT_3, T_4 and T_3

Fig. 1A indicates that TETRAC is not bound by hemoglobin, in contrast to T_4 (Fig. 1C), T_3 (Fig. 1D) and rT_3 (Fig. 1B). Thus, the alanyl amino group is required for the iodothyronine–hemoglobin interaction.

Effect of pH on hemoglobin–thyroid hormone interaction

The pH of hemoglobin solutions was varied over the range of 6.0–10.0 and hemoglobin-binding of labeled T_3 and T_4 was measured (Table I). Gemmill[7] has shown and Handwerger *et al.*[8] have confirmed that the p*K* of the phenolic hydroxyl group of T_3 is *ca.* 8.3, whereas that of T_4 is *ca.* 6.6. If ionization of the hydroxyl site were important to hormone-binding by hemoglobin, we would expect pH changes in the 6.0–8.0 range to affect T_3- and T_4-binding differentially. It is clear from Table I

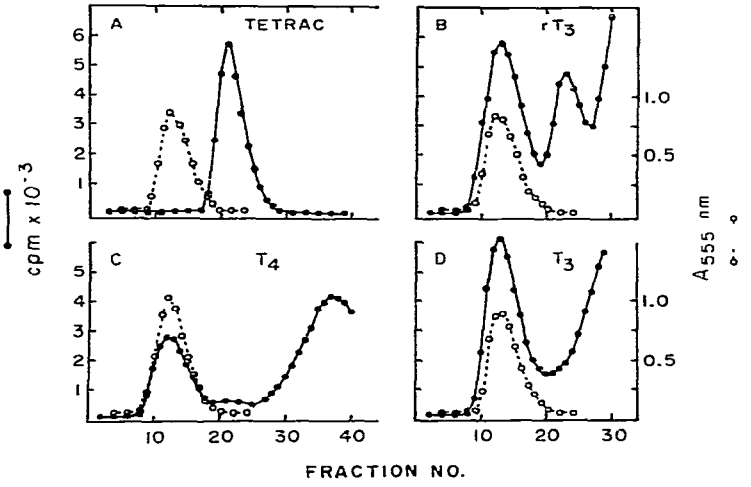

Fig. 1. Gel filtration (Sephadex G-100) of human hemoglobin, 1.0 g/dl 0.02 *M* phosphate buffer, pH 7.6, incubated for 24 h at 4°C with labeled thyroid hormone analogues: A, [^{125}I]-tetraiodothyroacetic acid (TETRAC); B, [^{125}I]-reverse T_3 (rT_3); C, [^{125}I]-thyroxine (T_4) and D, [^{125}I]-triiodothyronine (T_3). Emergence of hemoglobin from columns was monitored spectrophotometrically at 555 nm. Co-elution of hemoglobin fraction and labeled rT_3, T_4 and T_3 is demonstrated; TETRAC does not elute with hemoglobin. Column dimensions were 30 × 1.5 cm, fraction volume was 1 ml, void volume included tubes 1–9. Panel B shows elution of contaminating radioiodide (tubes 21–25) and of unbound rT_3 (tubes 26–30). Panel C shows elution of unbound T_4 (tubes 27–40) and panel D reveals emergence of unbound T_3 in tubes 21–30.

TABLE I

EFFECT OF pH ON BINDING OF IODOTHYRONINES BY HUMAN HEMOGLOBIN (Hb)
t = Calculated by paired *t*-test; NS = not significant.

	Fractional binding of hormone by Hb		
	pH 6.0	*pH 8.0*	*pH 10.0*
[^{125}I]T_4	0.066 ± 0.006*	0.142 ± 0.018	0.249 ± 0.160
[^{125}I]T_3	0.018 ± 0.004	0.130 ± 0.008	0.226 ± 0.038
t	6.76	0.62	0.32
P T_4 *vs.* T_3	<0.01	NS	NS

that pH influences hemoglobin-binding of these iodothyronines similarly, rather than differentially. T_3- and T_4-binding increase comparably at pH 8.0 and 10.0, while at pH 6.0 T_3 and T_4 at pH 6.0, however, is small (<7%). We conclude that the contribution of the thyroid hormone phenolic hydroxyl to the heme protein binding of iodothyronines is small or negligible.

DISCUSSION

The interaction of thyroid hormone and hemoglobin is a recently recognized phenomenon[1] that we have found to occur progressively with time in the intact human erythrocyte[3]. Once bound to hemoglobin, T_4 and T_3 are no longer detectable by radioimmunoassay[1] and the hemoglobin–thyroid hormone complex is one from which labeled iodothyronine is minimally displaceable by unlabeled thyroid hormone[1]. The qualities of non-displaceability of heme-bound hormone and lack of detectability of unlabeled hormone bound to hemoglobin precluded conventional ligand–protein analysis (such as determination of affinity constants) and mandated that the current studies of hemoglobin interactions be conducted only with labeled thyroid hormone and iodothyronine analogs.

The data presented here suggest that the amino group of the alanine side chain on iodothyronines is the principal site of interaction with hemoglobin. The alanyl carboxyl group cannot be primarily involved in the interaction. The fact that hemoglobin binds T_4, T_3 and rT_3 indicates that the 5- and 5′-iodines are not essential to binding. It remains to be established whether the thyronine structure is required for binding; conformational rigidity of the diphenyl ether structure, conferred by the 5-iodine[9], is obviously not needed. Because the 5′-iodine was also found to be unimportant to the interaction, we anticipated that the phenolic hydroxyl group of the iodothyronines would not relate to binding; the 5′-iodine is known to influence the p*K* of the hydroxyl group[7,10]. Studies of hormone-binding in the pH range of 6.0–10.0 confirmed that T_3- and T_4-binding were not differentially affected, indicating that ionization of the hydroxyl was unimportant to binding. The nature of the thyroid hormone amino group bonding to hemoglobin is unknown, but the fact that this interaction appears to involve the heme prosthetic group of hemoglobin[1] and that thyroid hormone may form apparently covalent complexes non-enzymatically with proteins[11] raises the possibility that an amide bond may develop between the amino group of hydrophobic thyroid hormone and an accessible carboxyl group (*e.g.*, propionyl carboxyl) of hydrophobic heme.

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