Vol. 124, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1984 Pages 29-36

THE BINDING OF THYROID HORMONE RECEPTORS TO DNA

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Received August 28, 1984

SUMMARY: The behaviour of tri-iodothyronine (T_3)- and thyroxine (T_4)-receptor complexes when bound to native DNA-cellulose is reported. Equal and large proportions of both T_3- and T_4-receptor complexes bind to DNA but although T_3-receptor complexes are 99% recoverable by 0.5 M NaCl buffer elution, only 60-70% of the T_4-receptor complexes are regained. The balance appears as free T_4, apparently released as the T_4-receptor complexes bind to the DNA whilst the corresponding receptor remains bound. This effect is independent of T_4-receptor complex/DNA ratio up to ca. 4 fmol/µg DNA, of the presence of an equal amount of unoccupied receptor and of an eight-fold concentration range of both T_4-receptor complex and DNA at a fixed ratio, in the cellulose matrix. Preformed receptor-DNA material, likewise, only accepts some 60% of the expected quantity of T_4 whereas the capacity for T_3 appears to be similar to that of free receptors.

Oppenheimer et al. (1) have shown that in hepatic cell nuclei the receptors are 50% occupied and that 85% of the total iodothyronine specifically bound is in the form of tri-iodothyronine (T_3) with the remaining 15% as thyroxine (T_4) (2). Therefore, although T_4 is known to stimulate growth and development and to increase the levels of several enzymes, it is generally believed that these hormonal effects are attributable to T_3 generated by monodeiodination of T_4 in the peripheral tissues (3-5). Because this conversion accounts for approximately 80% of the daily T_3 production and because the biological activity of T_3 is about five times greater than T_4, all the apparent T_4 activity could be accounted for by conversion to T_3 (5-6). Accordingly, T_4 has become to be regarded as a prohormone to T_3 with little intrinsic nuclear activity itself (2-4, 7). However, recent evidence suggests that such an interpretation of the biochemical role of T_4 may be an oversimplification. There are a variety of clinical states, in which the physiological condition conforms more closely to the serum concentration of T_4 than to the concentration of T_3, suggesting that T_4 does have intrinsic biological activity (4). Samuels et al. (8) have shown that T_4 increases the rate of growth in GH_1 cells by three fold in the absence of T_4- to T_3- conversion. Yoshimasa and Hamada (9) have reported that when T_4 was administered to hypothyroid rats together with iopa-

0006-291X/84 $1.50

noic acid, to block T_4 to T_3 conversion, all the iodothyronine, which bound to the hepatic nuclear receptors, was in the form of T_4 and in an amount comparable to that found when the rats were given T_4 alone. Nevertheless, the hepatic DNA content was found to be increased whilst the α-glycerophosphate dehydrogenase activity was elevated three-fold. These studies clearly indicate that although T_4 is a prohormone, it may also be an active hormone.

In an attempt to understand the mechanism of action of T_4, we investigated the binding of T_4-receptor complexes (T_4R) to DNA and also the binding of T_4 to preformed unoccupied receptor-DNA complexes. These studies were compared with identical experiments run in parallel in which T_3-receptor complexes (T_3R) were used. The results indicate that when receptor protein is bound to DNA all of the receptor sites can bind T_3 but that some 35% of the sites are unavailable to T_4.

MATERIALS AND METHODS

Reagents: L-3,3',5'-[^{125}I] triiodothyronine (3380 μCi/μg) and L-[^{125}I] thyroxine (1250 μCi/μg) were from New England nuclear and unlabelled T_3 and T_4 obtained from Sigma Chemical Co. The iodide content of the [^{125}I]-labelled hormones was routinely monitored by the method of Green (10) and for [^{125}I]T_3 was 3-5%, on a count basis, and for [^{125}I]T_4 it did not exceed 12%. Native DNA-cellulose came from P.L. Biochemicals.

Buffers: Buffer A, 10 mM Tris-HCl, 0,25 M sucrose, 1 mM $MgCl_2$, 2 mM DTT, and 400 mM KCl, pH 7.8 at 2°. Buffer B, 10 mM sodium phosphate at pH 7.8 containing 0,1 mM EDTA, 2 mM DTT, 10% (v/v) glycerol and 25 mM NaCl.

Preparation of nuclear extract: Triton X-100 washed nuclei were isolated from the livers of male Wistar rats (200-250 g) as described previously (11). The nuclear receptors for the thyroid hormones were extracted using the method of Ricketts et al. (12) except that the nuclei were extracted with buffer A and the solubilized protein dialysed against buffer B.

Hormone binding assays: For hormone binding to receptors nuclear extract was charged with [^{125}I]T_3 or [^{125}I]T_4 at 3°C for 18 h in buffer B at a final volume of 1 ml. The hormone concentrations used ranged from 1.25 - 3 nM for [^{125}I]T_3 and 0.8 - 7.5 nM for [^{125}I]T_4. The receptorbound and free hormone were separated on Sephadex G25(Fine) columns as previously described (12). Non-specific hormone binding was determined in the presence of a 2000-fold excess of unlabelled hormone and has been subtracted from the total binding values. The [^{125}I] hormone-receptor complexes were utilized within 10 min after separation from free hormone.

DNA-affinity chromatography: The DNA binding of hormone-receptor complexes was investigated by DNA-cellulose affinity chromatography. Nuclear extract (1 ml), containing either [^{125}I]T_3- or [^{125}I]T_4-labelled receptors, was applied to 1 ml DNA-cellulose columns (1.5 x 1.0 cm, 250 μg DNA) at a flow rate of 2 ml/h and at 4°C. The columns were further eluted at 12 ml/h with buffer B and after 30 fractions a 50 - 300 mM NaCl linear gradient was started in order to release DNA-bound receptors. Fractions of 1.2 ml were collected and counted in an Auto-gamma spectrometer (efficiency about 70%).

Hormone-protein complexes not binding to DNA co-eluted with free hormone in the void volume. To separate these components, all later experiments used columns of 0.25 ml DNA-cellulose (300 μg DNA) underlayered with 0.5 ml Sephadex G25(Fine) and these were found to give excellent resolution (Fig. 1). Routinely, 1 ml nuclear extract containing either [^{125}I]T_3- or [^{125}I]T_4-labelled receptors was applied to the column at a flow rate of 5 ml/h and eluted with buffer B at 12 ml/h until a total of five fractions was collected.

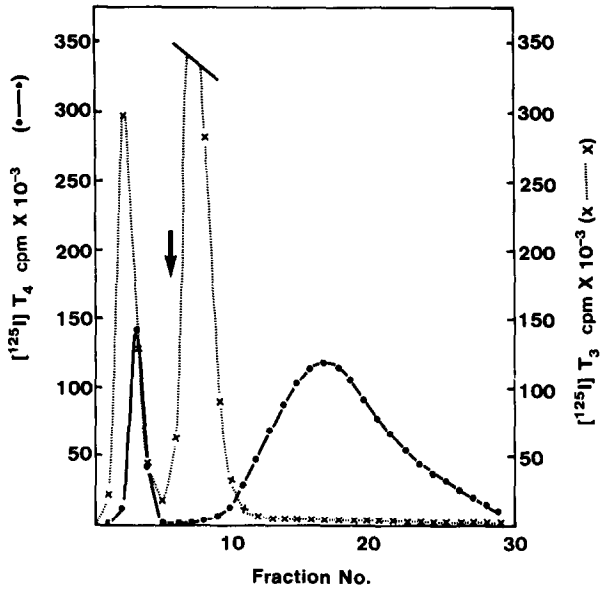

Fig. 1 Resolution of hormone-receptor complexes and free hormone on DNA-cellulose columns underlayered with Sephadex G25(Fine). [^{125}I]T_3R complexes and [^{125}I]T_4 were chromatographed separately and as described in "Materials and Methods". The arrow indicates the application of 0.5 M NaCl buffer.

This was followed by elution with buffer B containing 500 mM NaCl until over 90% of the radioactivity was recovered. Fractions of 1 ml were collected and counted for [^{125}I]-labelled hormone. From Fig. 1 it is observed that hormone-protein complexes not binding to DNA eluted in fractions 2-3, hormone-receptor complexes which bound to DNA eluted in fractions 6-9 and that free hormone eluted in fractions 11-23. Because the elution of free hormone took some 12 fractions, a modified procedure was introduced in later experiments by which after fraction 11 further elution was made with a mixture of methanol and ammonia (3:1 v/v) and this affected a 95% recovery of the free hormonein 4-5 fractions. For every experiment control columns, consisting of 0,25 ml cellulose underlayered with 0,5 ml Sephadex G25(Fine), were run in parallel and eluted in an identical manner to the DNA-cellulose columns. Radioactivity counts in the control column fractions, which corresponded to hormone-receptor complex and free hormone elution from the DNA-cellulose columns, were subtracted from those for the experimental columns.

Hormone-receptor complexes, which could only be eluted with salt concentrations above 200 mM from DNA-affinity columns, were also released by DNase I hydrolysis indicating that these complexes were initially bound to DNA.

RESULTS

The elution profiles of [^{125}I]T_3R and [^{125}I]T_4R from DNA-cellulose columns

Typical results from the DNA-affinity chromatography of thyroid hormone-receptor complexes are shown in Figs. 2(a), (b). The void-volume peak from T_3R elution has a relatively small part of the applied material whereas from T_4R chromatography this fraction contains the major part of the radioactivity. Analysis of the void volume fractions on Sephadex G25(Fine) columns showed

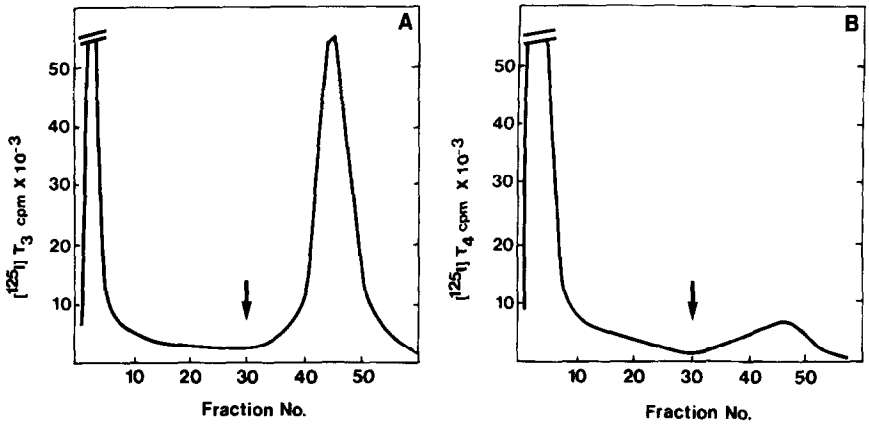

Fig. 2 Affinity chromatography of T_3- and T_4-receptor complexes on DNA-cellulose. Chromatography was performed as described in "Materials and Methods". The arrow indicates the start of a linear 50-300 mM NaCl gradient.

that the major part of the $[^{125}I]T_3$ activity was protein-associated with only a very small amount of free $[^{125}I]T_3$. The corresponding $[^{125}I]T_4$ charged fraction, whilst yielding a lesser amount of protein-associated $[^{125}I]T_4$ was found to have a large quantity of free $[^{125}I]T_4$. Referring again to Figs. 2(a), (b) it is clear that the major part of the applied $[^{125}I]T_3R$ was bound to the DNA and released by the salt gradient whereas only a small part of the $[^{125}I]T_4$ was recovered in this way. Each of these fractions was charged with $[^{125}I]T_3$ at concentrations known to give >90% occupancy and it was found that the total receptors present in both were the same. Therefore, substantial amounts of unoccupied-receptor must have been present in the salt-released $[^{125}I]T_4R$ fraction.

It is concluded that whilst T_3R binds strongly to DNA with little dissociation of T_3, in the case of T_4R an equally large binding results in the release of a substantial fraction of T_4, the receptor remaining bound to DNA. From Figs. 2(a), (b) two more conclusions can be drawn, first that the free T_4 appears in the void-volume fraction before application of the salt-gradient so that the dissociation of T_4 cannot be attributed to an effect of increased ionic strength. Second, the shape of the void-volume peak (Fig. 2(b)) shows no indication of a slow release of T_4 from the DNA-bound T_4R; the dissociation must be about synchronous with the binding.

These conclusions were reinforced by further experiments in which the fractionation of a large quantity of $[^{125}I]T_4R$ applied to a DNA-cellulose-Sephadex column was compared to the behaviour of a comparable amount of $[^{125}I]T_4$. The dissociated hormone eluted in exactly the same way as $[^{125}I]T_4$ added directly to the column.

TABLE 1 DNA-affinity chromatography of thyroid hormone-receptor complexes (T_3R and T_4R)

Fraction	T_3R fmol				T_4R fmol			
	DNA col.	control col.	net.	% of net applied	DNA col.	control col.	net.	% of net applied
applied	673.5	676.5	646.9	-	673.5	637.5	540.8	-
unbound complex	78.6	646.9	75.2	11.6	54.7	540.8	59.5	11.0
bound complex	575.0	8.3	566.7	87.6	323.2	31.0	292.2	54.0
free hormone	22.1	7.1	5.0	0.8	291.5	102.4	189.1	35.0

DNA = 300 µg/column. As the actual amounts of T_3R and T_4R applied were not exactly equal the various column fractions were normalised to the quantity of T_4R (673.5 fmol) applied to the control column. Total hormone recoveries on all columns >95%. The net. applied T_3R or T_4R is identified with the unbound complex found in the appropriate control column.

Quantitation of the interaction of thyroid hormone-receptor complexes with DNA-cellulose. Further experiments were made with DNA-cellulose and cellulose (control) columns underlayered with Sephadex G25(Fine). Receptors at >90% occupancy (1.25 nM [^{125}I]T_3 or 7.2 nM [^{125}I]T_4) were applied in 1 ml to DNA-cellulose and cellulose columns. The analytical results (Table 1) show that equal proportions of T_3R and T_4R were bound to DNA but whilst only 0.9% of the originally bound T_3R dissociated to T_3, in the case of T_4R the proportion dissociating is 39.3%, thus confirming the qualitative results reported above.

The enhanced dissociation of T_4R bound to DNA is shown (Table 2) to be independant of the concentration of both DNA and T_4R in the column when a fixed

TABLE 2 DNA-affinity chromatography of thyroxine-receptor complex at constant T_4R/DNA ratio

DNA µg/column	T_4R fmol net. applied	net. bound	T_4 fmol net. free	free T_4 x 100 / *total bound T_4
75	431.9	183.7	102.5	35.8
150	911.5	384.7	223.8	36.8
300	1 488.1	589.7	323.0	35.4
600	3 128.4	1 466.8	781.2	34.8

T_4R preparation (>90% occupancy) was applied to DNA and control columns in volumes sufficient to give a constant T_4R/DNA ratio (ca. 5 fmol/µg DNA)
*total bound T_4R = net. bound T_4R + free T_4.

TABLE 3 DNA-affinity chromatography of thyroid hormone-receptor complexes at increasing R/DNA ratio

T_3R fmol		T_3 fmol	T_3	T_4R fmol		T_4 fmol	T_4
applied	net. bound	free	free % *	applied	net. bound	free	free % *
84.3	56.8	0.4	0.7	80.9	39.5	15.0	27.5
170.3	118.3	1.5	1.3	162.1	78.0	29.2	27.1
351.1	238.4	4.8	2.0	329.1	159.1	50.8	24.2
545.9	300.6	6.5	2.1	498.3	200.8	75.0	27.2
1105.7	565.2	7.3	1.3	994.4	359.5	126.4	26.0

DNA = 200 µg/column. T_3R and T_4R preparations >90% occupancy. *calculated as in Table 2.

ratio T_4R/DNA (ca. 5 fmol T_4R/µg DNA) is used. The lower proportion (ca. 66%) of the applied T_4R which bound to DNA in these experiments is due to the higher column flow rate employed (5 ml/h) compared to that of 2 ml/h used for the experiments in the proceeding section; it is known (14, unpublished observations) that about 0.5-1 h is required for complete adsorption of T_3R or T_4R by DNA.

Similar results (Table 3) were obtained by the addition of increasing quantities of T_4R to DNA and control columns. Although the ratio T_4R/DNA was changed from ca. 0.4 through 5 fmol/µg DNA the percentage of the originally bound T_4R dissociating remained constant whilst the percentage of T_4R bound decreased, due to the flow rate effect noted above, from ca. 70% to 50%. For T_3R the percentage dissociating was very small although the percentage of T_3R binding to DNA was at the same level as for T_4R. The smaller proportion of total bound T_4R dissociation in this series of experiments, compared to those reported in Tables 1 and 2, is considered to be due to inherent differences between the compositions of the nuclear extract preparations.

The binding of T_3 and T_4 to pre-formed receptor-DNA complexes

From the results of the foregoing experiments it seemed likely that on charging pre-formed R-DNA complexes, with concentrations of [^{125}I]T_3 and [^{125}I]T_4 known to give >90% occupancy of receptor in solution, that T_3 would bind to a much larger extent than T_4. Table 4 shows the results from such an experiment in which the nuclear extract preparation, when charged with 2 nM [^{125}I]T_3, bound the hormone to the extent of 586 fmol/ml, including non-specific binding. After incubation of 1 ml samples of uncharged nuclear extract with 200 µg DNA in cellulose columns, the void-volumes recovered were charged with 2 nM [^{125}I]T_3, indicating that 156 fmol of receptor were not bound (this includes the non-specifically bound T_3). Consequently 430 fmol of receptor had bound to

TABLE 4 T_3 and T_4 binding to pre-formed receptor-DNA complexes

	DNA-cellulose*	
	column 1	column 2
	fmol	fmol
1 ml uncharged nuclear extract applied**	585.5	585.5
non-binding fraction**	157.0	155.0
DNA-bound receptors	428.5	430.5
hormones added	7.2 nM [^{125}I]T_4	2 nM [^{125}I]T_3
receptors desorbed**	145.0	138.0
net. DNA-bound receptors charged	283.5	292.2
T_4R released by 0.5 M NaCl	145.0	-
T_3R released by 0.5 M NaCl	-	239.7

* DNA = 200 µg/column

** assayed by 2 nM [^{125}I]T_3 charging

DNA, the quantity now not including non-specific binding capacity as the void-volume would contain all of the non-receptor protein (13). After charging the R-DNA-cellulose complexes with, respectively, 7.2 nM [^{125}I]T_4 and 2 nM [^{125}I]T_3 the columns were eluted to collect a void-volume containing desorbed receptors, which by charging with 2 nM [^{125}I]T_3 were found in each case to amount to about 150 fmol. It appeared, therefore, that about 280 fmol of DNA-complexed receptor had been available for charging but, on elution of these with 0.5 M NaCl buffer, 145 fmol of T_4R and 240 fmol of T_3R were recovered i.e. a fairly large fraction of the DNA-bound receptors had not been accessible by T_4. As the quantity of receptors in each of the columns had been identified throughout by [^{125}I]T_3 the proportion of them available to T_4 is taken (Table 4) to be 60,5%. The same parameter, calculated from the data of Table 1, is equal to 61,7%.

DISCUSSION

Native DNA has been found to bind T_3R or T_4R equally over a range of 60-90% of the applied complexes and up to 5 fmol/µg DNA. In agreement with other reports (14, 15) it is found that DNA-bound T_3R is stable and 99% recoverable by elution with buffered 0.5 M NaCl. By contrast, only some 60-70% of DNA-bound T_4R is regained, the deficit appearing as free T_4, apparently released as T_4R binds to DNA, whilst the now unoccupied receptors remain bound and co-elute with the unaffected T_4R. An increase in the K_D for T_4R bound to DNA cannot be invoked to explain the phenomenon as it would require a progressive loss of T_4; such a time-dependent loss was not observed.

Of our experiments only those on the binding of T_3 to pre-formed receptor-DNA complexes might indicate an increase of capacity as reported by Inoue et al. (15). The data (Table 4) show a final recovery of T_3R to be 82% of the apparently available receptors. This is sufficiently within the range of 70-90% of the available T_3R found to be complexed with DNA (Tables 1, 2) as to suggest that the quantity of uncharged receptors accepted by DNA from nuclear extract was equal to that expected from the initial receptor assay. Had there been any generation of receptor sites, by the action of DNA on nuclear extract, of the magnitude reported by Inoue et al. (15), then the final recovery of T_3R (released by buffered 0.5 M NaCl) would have been greater (calculated from data, (15) as 410 fmol) than that found (240 fmol).

Although the reduced T_4 binding to pre-formed receptor-DNA complex could be explained in terms of an increase of K_D, because it resulted from an equilibrium charging system, as already discussed this interpretation cannot be made for the converse experiments. Because both kinds of experiments indicate a similar deficiency of T_4 binding to DNA-bound receptor it is concluded that whilst the major fraction of sites bind T_4 tightly, a substantial proportion has little affinity for this hormone.

REFERENCES

1. Oppenheimer, J.H., Schwartz, H.L. and Surks, M.I. (1974) Endocrinology 95, 897-903.
2. Surks, M.I. and Oppenheimer, J.H. (1978) J. Clin. Invest. 60, 555-652.
3. Schwartz, H.L., Surks, M.I., and Oppenheimer, J.H. (1971) J.Clin. Invest. 50, 1124-1130.
4. Ingbar, S.H. and Braverman, L.E. (1975) Annu. Rev. Med. 26, 443-449.
5. Chopra, I.J., Solomon, D.H., Chopra, U., Wu, S.Y., Fisher, D.A. and Nakamura, Y. (1978) Recent Prog. Hormone Res. 34, 521-556.
6. Ramsden, D.B. and Hoffenberg, R. (1983) Clin. Endocrinol. Metab. 12, 101-115.
7. Surks, M.I., Schadlow, A.R., Stock, J.M. and Oppenheimer, J.H. (1973) J. Clin. Invest. 52, 805-811.
8. Samuels, H.H., Stanley, F. and Casanova, J. (1979) J. Clin. Invest. 63, 1229-1240.
9. Yoshimassa, Y. and Hamada, S. (1983) Biochem. J. 210, 331-337.
10. Green, W.L. (1972) J. Chromatogr. 72, 83-91.
11. Samuels, H.H., Tsai, J.S., Casanova, J. and Stanley, F. (1974) J. Clin. Invest. 54, 853-865.
12. Ricketts, M.H., De Wet Groenewald, J. and Wilson, B.D. (1980), S.A. J. Sci. 76, 377-378.
13. Wium, C.A. (1981) Thesis for M.Sc. degree, University of Stellenbosch.
14. MacLeod, K.M., and Baxter, J.D. (1976) J. Biol. Chem., 251, 7380-7387.
15. Inoue, A., NaKagawa, K. and Morisawa, S. (1981) Eur. J. Biochem., 114, 509-516.