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Thyroid hormone binding by a component of mitochondrial membrane

(thyroxine/triiodothyronine/mitochondrial membrane protein/hormone receptors/hormone action)

KENNETH STERLING AND PETER O. MILCH

Department of Medicine, Columbia University College of Physicians and Surgeons, and Protein Research Laboratory, Bronx Veterans Administration Hospital, Bronx, New York 10468

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ABSTRACT **The thyroid hormone, triiodothyronine, has been shown to be bound by the intranuclear chromatin protein associated with active DNA, where it is believed to stimulate transcription. Evidence exists that the thyroid hormones have direct action not only on nuclei, but also on mitochondria. Therefore, specific proteins that bind thyroid hormones in the mitochondria should be demonstrable.**

Mitochondria were isolated from homogenized rat livers by sedimentation through 0.25 M sucrose solution, followed by washing four times to free them of microsomes. Strong binding of thyroid hormones was observed in mitochondrial fractions prepared from both the membranes and the matrix. After incubation in an ice bath with increasing amounts of triiodothyronine with added tracer [^{125}I]triiodothyronine, the matrix infrequently contained specific saturable receptor sites, but usually exhibited strong "nonspecific" interaction. In contrast, a protein fraction obtained from the mitochondrial membranes revealed on Scatchard plot an association constant approximating 4×10^9 liters/M, significantly higher than that we have obtained for isolated rat liver nuclei (about 5×10^8 liters/M). Partial purification of the mitochondrial membrane protein resulted in pronounced diminution of "nonspecific binding" and a higher apparent association constant (k_a greater than 10^{11} liters/M).

The demonstration of a triiodothyronine binding protein in the mitochondrial membrane is consistent with direct hormone action upon mitochondria. The binding sites of nuclei and mitochondria both exhibit saturability, and are considered to have a role in hormone action. The nuclear protein binding is believed to influence growth, development, and functions concerned with cell maintenance, whereas mitochondrial protein binding is probably concerned with reversible effects on energy metabolism.

The protein and peptide hormones have been shown to be bound by the plasma membranes of target cells and to activate adenylate cyclase, a membrane-bound enzyme (1). In contrast, the small molecule steroid hormones penetrate the cellular plasma membrane where they are bound to cytosol receptor proteins. These cytosol receptors with bound steroid hormones have been shown to be translocated to the nucleus where they enhance DNA transcription (2, 3). Recently, for example, Schutz, Beato, and Feigelson (4) have shown that cortisol increases the amount of messenger RNA (mRNA) that directs the synthesis of a specifically inducible hepatic enzyme (tryptophan oxygenase) in the livers of cortisol-treated rats.

Numerous recent reports have described subcellular binding proteins of thyroid hormones concerned with putative mechanisms of hormone action at the cellular level (5–30).

The proposed model attributes a rather different role to the cytosol binding proteins than is the case with steroid hormone action. No reports have described translocation of specific cytosol receptor proteins to the nucleus; moreover, the observations thus far signify the contrary. Indeed, studies two years ago in our laboratory indicated appreciable uptake of [^{125}I]triiodothyronine *in vitro* at 4° or 37° by rat renal cell nuclei isolated by centrifugation through 2.4 M sucrose solution. We found the nuclear uptake was invariably greater with nuclei suspended in saline solution or potassium phosphate buffer than with nuclei resuspended in cytosol prepared from the very same rat kidneys, or in human serum albumin at 1% protein concentration, about the same as that of cytosol. This observation, subsequently confirmed by Oppenheimer's group (15), has suggested that free hormone rather than receptor-bound hormone may enter effector loci such as the cell nucleus, in contrast to the postulated model of steroid hormone action.

The many recent reports from the laboratories of Oppenheimer (8–15), Samuels (16–20), and de Groot (5–7) provide evidence of specific nuclear receptors for the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), and suggest that the cell nucleus is a significant locus of hormone action. Indeed, as early as 1966, Siegel and Tobias demonstrated the nuclear localization of thyroid hormone by radioautography of tritium-labeled hormone added to cell cultures of human renal epithelial cells, grown in monolayer (31, 32), an observation which forecast the later developments. These authors showed a diminution of the number of tritium grains over the nucleus if actinomycin D had been added to the cell cultures, but an increase after addition of puromycin, a finding compatible with thyroid hormone action at the transcriptional level in the nucleus.

Prior to and throughout the same period of time, however, Tapley and his associates (33–42) have been accumulating evidence that suggests direct thyroid hormone action upon the mitochondria of responsive cells. Binding of thyroid hormones by mitochondrial membranes was confirmed by Tata, Ernster, and Suranyi, who reported microsomal and nuclear binding as well (43). Perhaps the most striking observation has been the demonstration of increased mitochondrial protein synthesis in as short an interval as 3 min after the addition of T_4 or T_3 to isolated rat liver mitochondria *in vitro* (35).

Recently we have been studying the binding of T_4 and T_3 to cell proteins (23, 24). The present communication concerns the finding of a protein fraction of the mitochondrial membrane which binds T_4 and T_3 in the physiological range, and is presumed related to hormonal effect upon energy metabolism.

After isolation of mitochondria from rat liver and kidney, the binding of labeled hormones by intact mitochondria was studied, followed by examination of the mitochondrial matrix and membranes, and subsequently protein fractions extracted from the mitochondrial membranes.

Abbreviations: T_3, triiodothyronine; T_4, thyroxine.

MATERIALS AND METHODS

The isolation and experimental handling of nuclei and cytosol have been described (22–24).

Mitochondria were isolated from rat livers and kidneys by adaptation of the method of Buchanan and Tapley (35). Male Sprague–Dawley rats, weighing 300 g or more, were used in all experiments. Surgically thyroidectomized rats were purchased from Hormone Assay Labs., Chicago, Ill., and were used at least 1 month after the operation. The animals were killed by a single blow to the head. Livers and/or kidneys were immediately removed, weighed, and placed on ice. All subsequent procedures took place at 0–4°. The tissues were minced with scissors and a 10% homogenate was prepared in 0.25 M sucrose with a glass homogenizer vessel and motor-driven Teflon pestle (Eberbach Corp., Ann Arbor, Mich.) The homogenate was centrifuged for 10 min at 2250 × *g* in a Sorvall RC 2-B refrigerated centrifuge. The resulting pellet, consisting mainly of nuclei and rough cellular debris, was discarded and the supernatant was recentrifuged at 9000 × *g* for 10 min. The resulting pellet consisted of mitochondria contaminated with microsomes. The microsomal contamination was removed by resuspending the pellet in 10 ml of 0.25 M sucrose, centrifuging for 10 min at 9000 × *g*, and repeating the procedure three times, a total of four washes, after which the final supernatant was clear. The mitochondrial pellet was resuspended in 2 ml of tris(hydroxymethyl)aminomethane-chloride/ethylene diamine tetraacetic acid buffer (50 mM Tris·HCl/10 mM EDTA), pH 7.0, and sonicated with six 15-sec bursts at 30-sec intervals with a Savant Instruments Model no. 1000 Insonater. The sonicated material was centrifuged for 1 hr at 140,000 × *g* in a Beckman Spinco L3-50 ultracentrifuge in a SW-40 rotor. The resulting supernatant, representing the mitochondrial matrix, was diluted to correspond to 1 ml of mitochondrial matrix per 1 g of tissue.

Alternatively, the pellet, consisting of membrane fragments, was resuspended in a small amount of pH 7.0 Tris/EDTA buffer containing 0.5% Triton X-100 and incubated for 10–15 min at 0°. It was then diluted to the same extent as described for the matrix and recentrifuged for 1 hr at 140,000 × *g*. The resulting supernatant was termed "mitochondrial membrane protein."

Aliquots (0.4 ml) of either matrix or mitochondrial membrane protein were incubated for 1 hr with gradually increasing levels (1.5 to 150 × 10^{-12} M) of T_3 or T_4 with tracer levels of [^{125}I]T_3 or [^{125}I]T_4 at 0°. During the incubation, each sample was briefly removed from the ice-bath, and radioactivity was determined for 30 sec to establish the total radioactivity present. Incubation was terminated by the addition of 0.2 ml of Dextran-coated charcoal (Norit "A" + Dextran 60,000–90,000 molecular weight, both 10 g/liter). The sample was mixed well on a Vortex mixer, let stand for 10 min at 0°, and finally centrifuged for 10 min at 10,000 × *g*. The supernatant was decanted, and radioactivity of both supernatant (bound hormone) and charcoal pellet (free hormone) was determined in a Packard Auto-Gamma Spectrometer. Association constant (k_a) was determined by plotting bound/free hormone (B/F) against bound hormone as a Scatchard plot (44, 45).

Aliquots of sonicated mitochondria labeled with tracer levels of [^{131}I]T_3 were subjected to paper electrophoresis in a 12.5 mM sodium borate buffer system at pH 10.0. Electrophoresis was carried out for 3.5 hr at 12 mA (constant current) at room temperature (22°). The dried paper strips were scanned with a Nuclear-Chicago Actigraph III strip scanner (Model 1004) and compared with strips of serum proteins run simultaneously, and stained with bromphenol blue after scanning.

Initial studies of the mitochondrial membrane protein were undertaken to free the specific receptors from nonspecific protein binding sites.

Gel filtration of mitochondrial membrane protein was carried out on a calibrated column of Sephadex G-200, 90 × 1.5 cm, at a flow rate approximating 15 ml/hr with 0.05 M Tris·HCl buffer, pH 7.0, and 3-ml fractions were collected for determinations of absorbance and radioactivity, and other analytic procedures.

[^{125}I]T_3 and [^{125}I]T_4 were obtained from Abbott Labs., North Chicago, Ill. [^{131}I]T_3 was purchased from Industrial Nuclear Co., St. Louis, Mo. Purity was checked by paper electrophoresis and paper chromatography.

Charcoal (Norit "A") was obtained from Amend Drug & Chemical Co., Irvington, N.J. Dextran was purchased from Pharmacia Fine Chemicals, Piscataway, N.J.

All other chemicals were obtained from commercial sources and were of the highest purity available.

RESULTS

Electrophoresis of sonicated mitochondria (Fig. 1) showed a single protein peak that did not correspond to any of the known serum binding proteins. Nuclear and cytoplasmic fractions gave results somewhat different in appearance (refs. 23 and 24, and work in progress—less mobility than mitochondrial protein).

Early experiments for the determination of the association constant using intact mitochondria or aliquots of mitochondrial matrix proved to be rather inconsistent and unpredictable. Only a small percentage (about 20%) of 30 experiments performed on the matrix gave a negative slope from which k_a could be calculated. The majority of the experiments resulted in a positive or zero slope.

The second approach, utilizing a protein fraction extracted from the pelleted membrane fragments with Triton X-100, proved to be more consistent and reproducible. Fig. 2 represents a typical curve obtained by this method. In this figure mitochondrial membrane protein, obtained from rat liver mitochondria, was used with T_3 and tracer [^{125}I]T_3 as ligand. The curve is representative of a dozen successful experiments. The mean association constant is 3.9 ± 3.04 (SD) × 10^9 liters/M. A smaller number of experiments, in which the ligand was T_4 with the appropriate tracer, yielded k_a of 4.4 ± 2.0 (SD) × 10^9 liters/M. Mitochondrial membrane protein prepared from liver mitochondria of surgically thyroidectomized rats gave an association constant with T_3 of 1.4 ± 0.6 (SD) × 10^9 liters/M. The differences (T_3 compared with T_4 and euthyroid compared with thyroidectomized) are not statistically significant by Student's *t* test (46). Because of the large number of rat kidneys required to obtain sufficient material, only one experiment was performed on mitochondrial membrane protein obtained from rat kidney mitochondria. The k_a with T_3 was 9 × 10^{10} liters/M but this value is not considered final. The number of binding sites (n) per cell is calculated as approximately 2000, based on the value of 1 × 10^8 cells per g wet weight of rat liver (47, 48), and on protein determinations.

The first efforts at fractionation of mitochondrial membrane protein resulted in a substantial reduction of nonspecific binding, permitting more complete Scatchard plots. As illustrated in Fig. 3, which typifies the protein elution patterns, two peaks designated "A" and "B" were invariably found with rat liver mitochondrial membrane protein. Both

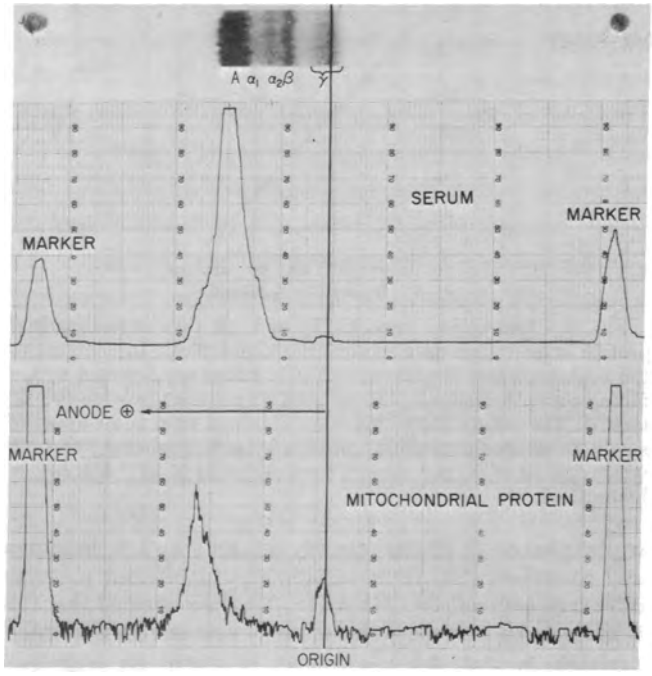

FIG. 1. Electrophoresis of thyroid hormone binding protein in rat liver mitochondria. Electrophoresis was performed on paper in sodium borate buffer, pH 10.0. The bromphenol-blue-stained paper strip of human serum proteins is illustrated at the top, with the protein bands appropriately labeled, A for albumin and α_1, α_2, β, and γ for the globulins. The small dots at each end of the strip were markers placed prior to scanning. The radioactive scan of human serum with added [^{131}I]T_4 was performed prior to staining of the paper strip, and shows a single large radioactive peak without resolution of prealbumin, albumin, and thyroxine binding alpha globulin. Lack of resolution of the serum thyroid hormone carriers is usual when serum is run in the strongly alkaline borate buffer system, which has been applied for resolution of subcellular hormone binding proteins (23). The lower scan of mitochondrial protein represents intact mitochondria that had been incubated with [^{131}I]T_3 (5.4×10^{-9} M) and applied at the origin, then subjected to electrophoresis. An insignificant radioactive peak is evident at the origin; the major peak has an appreciably higher mobility than that of any of the serum proteins.

exhibited radioactivity when [^{125}I]T_3 had been added prior to gel filtration; however, it was considered preferable to monitor by absorbance, without added radioactivity, so as not to interfere with subsequent binding studies.

The Scatchard plot illustrated in Fig. 4 represents the higher association constant anticipated with partial purification of mitochondrial receptor protein. The k_a exceeds 10^{11} liters/M.

The majority of the experiments reported here were performed on "fresh" material (i.e., on the day of the isolation of mitochondria and preparation of mitochondrial membrane protein). In some cases, however, enough mitochondrial membrane protein was prepared to perform the experiment the same day and to repeat it on a frozen and thawed aliquot the following day or several days later. Freezing and thawing did not alter the qualities of the mitochondrial membrane protein to any noticeable extent.

DISCUSSION

The inconsistent and erratic behavior of the mitochondrial matrix in the early experiments may be attributed to a large amount of nonspecific binding, virtually unsaturable, and an occasional minor amount of specific binding, saturable at variable levels. The fact that this small component, responsible for the specific binding, was present only in a minority (about 20%) of the series of 30 experiments, led us to believe that it was randomly destroyed during one of the preparative steps. The most likely place for such random destruction seemed to be the sonication step. While attempting to modify this part of the procedure, we considered an alternative hypothesis. Instead of random destruction, one can just as plausibly postulate random release. In other words, if the specific binders are attached to the membrane, a variable portion may get detached during the sonication procedure and end up with the matrix. The majority, however, presumably remains attached to the membranes, even after ultrasonic disruption of the mitochondria. Thus, focusing our attention on the membrane components, we succeeded in detaching these proteins by treatment with the nonionic detergent Triton X-100. Together with the desired protein component, a large amount of nonspecific binders, similar to those found in the matrix, are also released. For this reason, the Scatchard plot in Fig. 2 shows a negative slope only at lower concentrations of T_3. As the concentration of T_3 is increased (not illustrated), more and more nonspecific binders become involved and give the curve a flat or even rising appearance (i.e., zero or even positive slope). Amounts of T_3 as high as 200 μg/ml are bound to more or less the same extent as the highest experimental point shown at the extreme left. The reproducibility and marked consistency of the significant portion of the curve makes the extrapolation to the abscissa justified to estimate k_a and n, as done by classical Scatchard analysis (44, 45).

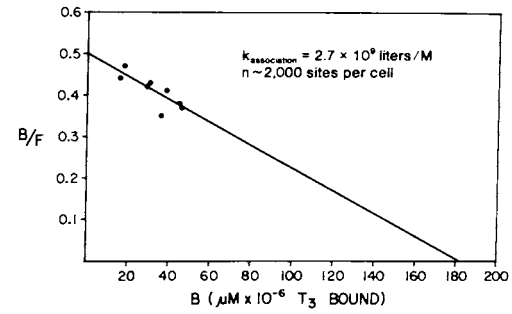

FIG. 2. Interaction between T_3 and rat liver mitochondrial protein (Scatchard plot). As explained in the *text* (see *Discussion*), the initial protein extracts contained sufficient nonspecific binders to flatten the curve with successive increments of T_3 beyond those illustrated. The points illustrated represent data without correction for "nonspecific binding," since the addition of a large excess of the ligand resulted in values approximating the highest experimental points illustrated. B/F = bound/free.

The more complete Scatchard plot of Fig. 4, with a higher association constant and lower nonspecific binding, resulted from partial purification. With marked reduction in nonspecific binding, the data were regarded as a more reliable indication of the properties of the mitochondrial membrane protein.

The further analytic work on the membrane binding protein is continuing at present, and further details are to be published elsewhere (K. Sterling and P. O. Milch, to be submitted). Studies with congeners of the thyroid hormones have been compatible with a relation between their binding affinity as ligands and their biological activity *in vivo*.

Considerable additional work will be required to elucidate fully the molecular mechanism of thyroid hormone action. For the present, it may be justifiable to suggest that more than a single locus of action may exist within responsive cells. On general descriptive biological grounds it is reasonable to speak of a duality of thyroid hormone action. One

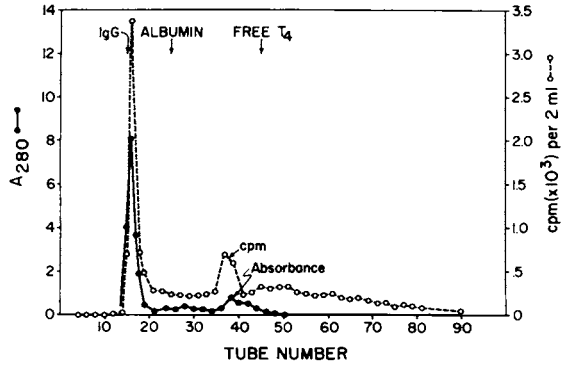

FIG. 3. Gel filtration of mitochondrial membrane protein on Sephadex G-200. The calibrations of the 90 × 1.5 cm column had previously been established with human serum with added [^{125}I]T_4, and are indicated in the figure. The initial sharp peak ("A") invariably emerged early, approximately one tube after the IgG peak of human serum; hence it was judged to represent one or more large protein molecules that bind T_3. The smaller "B" peak usually had its maximum T_3 radioactivity and absorbance at about tube 37 or 38 as shown, signifying a much smaller protein or polypeptide that binds T_3. The mitochondrial membrane protein had been enriched with 5.7×10^{-10} M [^{125}I]T_3 prior to gel filtration.

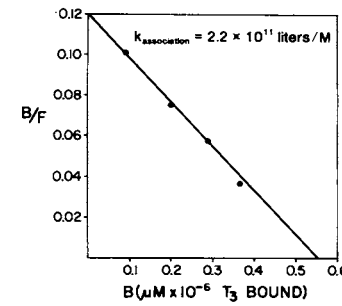

FIG. 4. Interaction between T_3 and rat liver mitochondrial protein after initial purification (Scatchard plot). In contrast to the Scatchard plot illustrated in Fig. 2, points are depicted with a falling curve throughout. The association constant exceeds 10^{11} liters/M. The points illustrated were obtained after subtraction of small "nonspecific binding" obtained from duplicate tubes to which 200 μg of T_3 per ml had been added (3×10^{-4} M). B/F = bound/free.

action could be an essentially irreversible effect upon growth and maturation, most strikingly typified by tadpole metamorphosis, but doubtless having its counterpart in adult mammalian tissue metabolism. This growth function can most readily be conceived of as mediated by cell nuclei. A nuclear locus of action is supported by evidence already cited (5–21), including, of course, the analogy to steroid hormone action (2–4).

Attractive though it might appear to some to ascribe all thyroid hormone action to a single pathway (8–15), it seems impossible to omit consideration of a direct primary effect upon mitochondria. The mitochondria can readily be conceived of as a locus for a form of reversible thyroid hormone action, including the thermogenic action in homeothermic animals. Working with isolated mitochondria, Tapley's group (33–42) has shown a number of changes of possible physiologic significance upon addition of thyroid hormone, including increased mitochondrial protein synthesis in as short an interval as 3 min after addition of T_4 or T_3 to isolated mitochondria *in vitro* (35). Moreover, Babior, Ingbar, and colleagues have shown increased entry of ^{14}C-labeled adenosine diphosphate into mitochondria after hormone administration (49).

The association constant we have obtained for the mitochondrial protein binding of T_3 greatly exceeds that observed for cell nuclei. Our initial k_a for mitochondrial membrane protein was slightly greater than 10^9 liters/M, and after initial purification and elimination of much nonspecific binding, the k_a was found to exceed 10^{11} liters/M. These values must be contrasted with our finding of k_a of 5×10^8 liters/M for the interaction between [^{125}I]T_3 and isolated rat hepatic nuclei (K. Sterling and P. O. Milch, to be submitted), a value that is similar to those reported in the literature (7, 13, 15) but which may become somewhat higher after further purification of the nuclear binding proteins. It may, therefore, be inferred that the earliest physiologic effects of thyroid hormone may well be due to mitochondrial effects upon energy metabolism. The effects mediated by the nuclei which involve new protein synthesis probably comprise more sustained thyroid hormone actions, which are evident after a lag period, as has been widely observed.

The thermogenic action of sodium-potassium adenosine triphosphatase (ATPase) recently reported by I. S. Edelman and coworkers (50, 51) is considered a significant pathway of energy expenditure. However, it has not seemed that this is likely to be a primary thyroid hormone action. Our interpretation is that this effect may represent an important "final common pathway" that requires nuclear and/or mitochondrial mediation for its occurrence.

In summary, the evidence presently available suggests a dual action of thyroid hormone: (*i*) an action upon nuclear transcription, concerned with growth, differentiation, and perhaps certain trophic functions, related to cell maintenance and (*ii*) an action upon mitochondria concerned with energy metabolism and thermogenesis. Our own findings show that upon entering the cell the thyroid hormones are firmly bound by cytosol receptor proteins, and subsequently by proteins of the nucleus and mitochondria which are both considered likely effector loci for hormone action.

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