THE EFFECT OF TRIIODOTHYRONINE ON THE PROTEINS OF LIVER MITOCHONDRIA

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1. Introduction

In young mammals thyroid hormones are required for growth [2,3]. Although the Na^+,K^+-ATPase [4] plays an important role in controlling oxygen consumption, and there may be a direct role of thyroid hormones in mitochondria, present evidence suggests that thyroid hormones exert their major effects by altering the rate of mRNA synthesis [5–7]. This seems to indicate that thyroid hormones control the rate of synthesis of many proteins. However, the rate of synthesis of only a small number of proteins has been shown to be markedly increased by thyroid hormone (serum albumin [8], malic enzyme [9], Na^+, K^+-ATPase [10], cytochrome *c* [11], growth hormone in the pituitary [7,12–13]). In some instances only an increased level of a particular protein or enzymatic activity after thyroid hormone administration has been seen (mitochondrial α-glycerophosphate dehydrogenase [9] and mitochondrial cytochrome oxidase [14]).

The development of two-dimensional gel electrophoresis [1] provides an opportunity for judging whether the action of thyroid hormone is confined only to a few proteins, to a modest number of proteins, or to very many. Hence we have examined the results of the administration of large doses of triiodothyronine in hypothyroid rats in the two-dimensional gel patterns from rat liver. Here, we discuss the results seen in mitochondrial fractions.

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2. Materials and methods

2.1. *Chemicals*

Several different batches of ampholines were obtained from LKB. Acrylamide, *N,N'*-methylenebisacrylamide and Coomassie brilliant blue, R-250 were obtained from Bio Rad Labs. and *N,N,N',N*-tetramethylethylene-diamine (TEMED) from Eastman Kodak. Nonidet P-40 (NP-40) and Sodium dodecyl sulfate (SDS) were manufactured by BDH Chem. Ltd. Ammonium persulfate and glycine (reagent-grade) were purchased from Fisher Sci. Co. and urea from Baker Chem. Co. 2-Mercaptoethanol (type I) and 3,5,3'-triiodo-L-thyronine, free acid, (T_3) were obtained from Sigma.

2.2. *Animals and treatments*

Male Sprague Dawley rats (~75 g) were obtained from Charles River Breeding Co., Wilmington, MA. As soon as they arrived in the laboratory, they were fed a low iodine diet and after 2 weeks, they were radiothyroidectomized (200 μCi ^{131}I, Mallinckrodt, St Louis, MO). The animals were kept on a Remington diet and used experimentally after stabilization of their body weight (~2 months). In these conditions, the sera of these rats contain ~18 ng T_3/100 ml and 0.7 μg T_4/100 ml (normal values are 70 ng T_3/100 ml and 3.9 μg T_4/100 ml).

These hypothyroidal animals were given T_3 (1 injection of 3 mg/100 g body wt) and sacrificed after 1, 2 or 3 days. After T_3 administration (53 h) the levels of T_3 and T_4 were 5779 ng/100 ml and 0.7 μg/100 ml, respectively, and after 4 days 2800 ng/100 ml and 0.4 μg/100 ml.

2.3. *Subcellular fractionation*

Subcellular fractions of rat liver were prepared according to [15]. This method yielded relatively uncontaminated mitochondrial and nuclear fractions. The mitochondrial fraction was sonicated in an ice bath for three 15 s bursts at 1 min intervals using a Branson Sonifier and then submitted to lysis buffer A in the experimental conditions described [1]. Proteins were estimated using the Lowry method [16] with bovine serum albumin as standard.

2.4. *Two-dimensional gel electrophoresis*

The two-dimensional gel electrophoresis of mitochondrial protein samples was performed as in [1] with the following modifications. Isoelectric focusing gels were made in glass tubing (130 × 4 mm i.d.). By this method, 100–150 μl sample containing 1–3 mg proteins could be applied and separated in the isoelectrofocusing electrophoresis. In SDS–electrophoresis, the running gel is ~30 cm high, the stacking gel is 4 cm and the spacers between the 2 glass plates are 1.5 mm thick. The acrylamide concentration in the running gel was 9%, and 4.5% in the stacking gel. The bis/acrylamide ratio was 1:36. A constant current of 25 mA was applied for each gel in the second SDS dimension for 7.5 h. All the isoelectrofocusing gels were run in SDS after equilibration as in [1].

The gels were stained in 0.1% Coomassie blue in 50% trichloroacetic acid or in 2.3% Coomassie blue in methanol, acetic acid, H_2O (45/45/10). Gels were destained in 7% acetic acid at 60°C and dried between two sheets of cellophane (Bio-Rad). The proteins used as molecular weight standards were: phosphorylase B (94 000); Lactoperoxidase (85 000); tubulin (55 000); ovalbumin (43 000); carbonic anhydrase (29 000); β-lactoglobulin (18 400); cytochrome *c* (13 400).

3. Results

Several fractions of rat liver were separated and fractionated. The proteins were extracted and analyzed on two-dimensional gel electrophoresis for comparison.

Figure 1 depicts the pattern obtained when 2 mg total homogenate protein was applied in the first, electrofocusing dimension. Using a mixture of ampholines pH 3.5–10 and pH 5–7 as described in

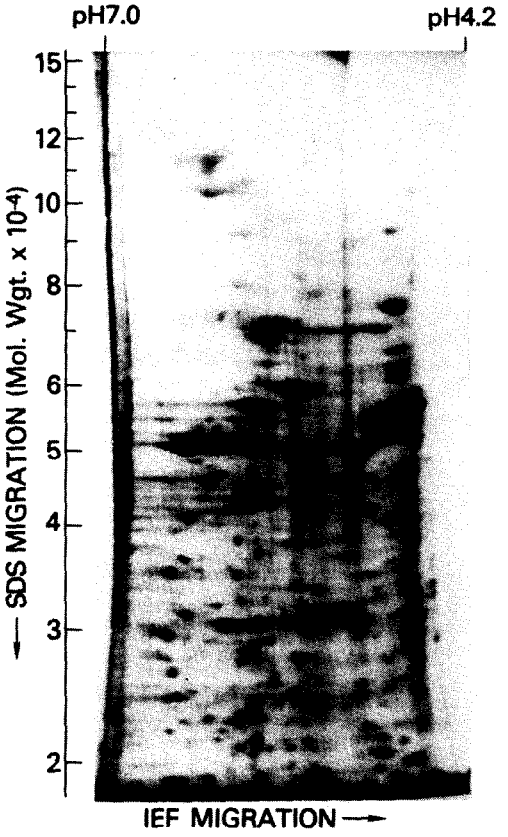

Fig.1. Two-dimensional gel electrophoresis of rat liver proteins after staining with Coomassie brilliant blue. 120 mg of fresh rat liver slices was sonicated and treated wih a lysis buffer A (1 ml) containing 9.5 M urea, 2% NP40, 1.6% ampholines pH 5 to 7, 0.4% ampholines pH 3.5 to 10 and 5% β MSH as described by O'Farrell [1] 150/μl of lysate containing about 3 mg of protein were submitted to isoelectric focusing and to SDS migration in the conditions in section 2.

O'Farrell's method [1], proteins with isoelectric points of 4–7 were well separated. In the second, SDS electrophoresis dimension, these proteins were separated according to their molecular weights from 20 000–120 000 after comparison with those used as molecular weight standards. In this pattern, 300–400 protein spots could be easily, and reproducibly, separated.

In fig.2A–C are shown the protein patterns obtained with the mitochondrial fraction using different ampholines to obtain the widest possible pH range.

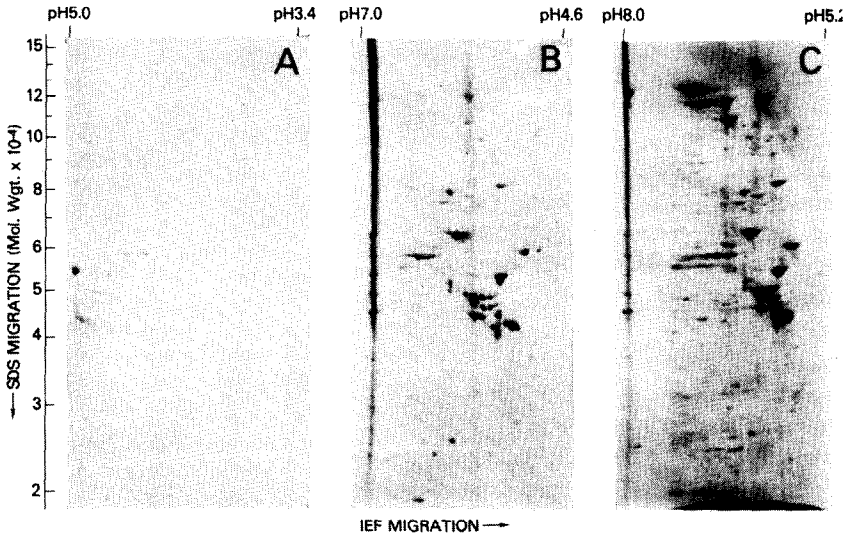

Fig.2. Distribution and separation of rat liver mitochondrial proteins in two-dimensional gel electrophoresis. The effect of ampholines and pH. About 1 mg mitochondrial proteins (100 μl) were applied. Isoelectric focusing (IEF) differed in the ampholine composition: (A) 0.4% (pH 3.5–10, 0.8%) (pH 2.5–4.0), ~0.8% pH 3.5–5, (B) 0.4% (pH 3.5–10), 1.6% (pH 5–7); (C) 0.4% (pH 3.5–10), 1.6% pH 7–9.

When acid ampholines (mixture of pH 2.5– 4.0 and 3.5–5 in equal volume were used (fig.2A) about 25 spots can be discriminated. The more acidic proteins had isoelectric points of ~3.5 and mol. wt 100 000, 60 000, 32 000 and 21 000.

In fig.1B, standard isoelectric conditions (ampholines pH 5–7) were used and in fig.2C, ampholines pH 7–9 were employed. Figures 2B,C show that in both of these situations 150 spots can be discriminated in the mitochondrial fraction with mol. wt 20 000–130 000. The resolution seems better in fig.2B when proteins are distributed between pH 6.6–7.0. At higher pH, the separation of basic proteins is not obtained. Such a discrepancy was noted in [1] and the numerous spots seen in the left sides of fig.2C,D probably represent basic proteins.

In fig.3 is compared the patterns of solubilized mitochondrial proteins from hypothyroid and T_3 injected animals. In this set of experiments, 2 mg protein was applied in the first electrofocusing dimension. Because of the protein load these patterns are somewhat different from those obtained in fig.2B. Nevertheless, comparison of fig.3A–D shows no major differences. More than 100 protein spots can be separated and the patterns are very similar in the 4 gels illustrated. However, among these protein spots, one difference can be noted. A protein of mol. wt ~94 000 and isoelectric point pH 4.8 appeared in fig.3B as indicated by the arrow, its concentration increased in gels of fig.3C,D. The same phenomenon has been observed in another set of experiments. The appearance of this spot seems related to the administration of T_3 since in fig.3A, no such spot could be detected. The dose of T_3 used was high but was chosen on the basis of the findings [9] that it causes maximum increases in some thyroid-sensitive proteins.

4. Discussion

Two dimensional gel electrophoresis, described first in [1,17] has been applied to the separation and the identification of proteins from different tissues, cells and bacteria [1,17–21]. This technique seemed useful to obtain an overall evaluation of the effects of thyroid hormones in protein synthesis in the liver. In our experiments, the O'Farrell method was slightly modified by using a larger isoelectric focusing gel and a longer SDS gel. Additionally, staining, destaining and final drying of the gel were modified. In these

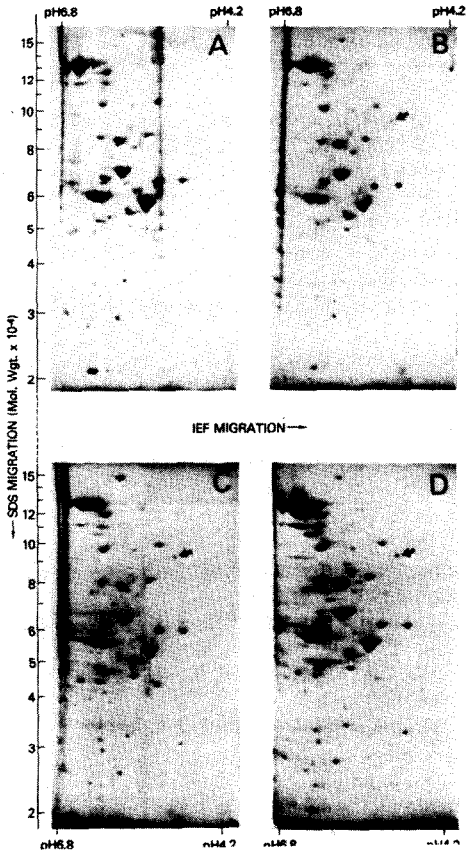

Fig.3. Two-dimensional gel electrophoresis of rat liver mitochondria proteins. The effect of triiodothyronine treatment. Mitochondria were prepared and treated as indicated in fig.2. 2 mg mitochondrial proteins in 150 μl lysis buffer A were applied on isoelectric focusing (conditions of fig.2B). The running gel conditions and treatments were described in section 2. (A) Hypothyroid control rat (see treatment in section 2). (B) Hypothyroid rat injected with 3 mg/100 g body wt with T_3 and sacrificed 1 day later. (C) The sacrifice is after 2 days. (D) The sacrifice is after 3 days.

conditions when isoelectrofocusing is performed with ampholines ranging from pH 5–7, it was possible to separate about 400 proteins in a whole homogenate (fig.1) and 150 proteins in the mitochondrial fraction (fig.2B,C) of liver. The patterns of the gels differed somewhat from one set of experiments to another depending in part on the amount of protein applied in the isoelectrofocusing dimension. Generally, good resolution was obtained when ~1 mg protein was analyzed using a mixture of ampholines pH 3.5–10 and 5–7 as in [1].

The protein components of different mitochondrial fractions (inner and outer membranes, soluble intermembrane space, matrix) in rat liver have been analyzed and characterized according to their molecular weights with SDS–polyacrylamide gels [22–25]. Generally, 50–60 protein species were separated.

After this work was completed an extensive analysis of the matrix proteins of mitochondria from a variety of different tissues was published [26]. They were able to differentiate ~140 proteins and identify, at least tentatively, 12 of them. Additionally, two further proteins of the inner membrane were identified [26]. Here, ~150 proteins can be reproducibly visualized in the whole mitochondrial fraction from liver. These proteins ranged from mol. wt 20 000–130 000 and presented isoelectric points varying from pH 4–7. The gel patterns seen in the present study are similar to those in [26]. Several reports note that large concentrations of thyroid hormone increase the rate of synthesis of protein by isolated mitochondria [27–30]. About 8–10 mitochondrial proteins are synthesized from mRNA transcribed from mitochondrial DNA. These comprise some of the subunits of cytochrome oxidase aa_3, $COQH_2$: cytochrome *c* reductase and the oligomycin-sensitive ATP synthetase [31]. None of these subunits are >40 000 mol. wt. Presumably only these proteins can be synthesized by isolated mitochondria. We are unable, however, to see any change in concentrations of these intrinsic mitochondrial proteins subsequent to the administration of triiodothyronine. Other mitochondrial proteins derived from nuclear transcription are increased by thyroid hormone. These include α-glycerophosphate dehydrogenase (mitochondrial) and cytochrome oxidase. α-Glycerophosphate dehydrogenase is not available in pure form and its subunit molecular weight is not available.

A major protein (mol. wt 54 000) of a mitoplast mitochondrial liver fraction was shown significantly decreased after thyroidectomy [24]. Here, we have visualized on two-dimensional gel electrophoresis, a major protein with mol. wt ~55 000, which could well represent the protein in [24]. In the absence of quantitative densitometery, it was not possible to be sure that the concentration of this protein was affected by hypothyroidism. Comparison of the

patterns of mitochondrial proteins (fig.3A–D) shows that one spot increased in size after T_3 treatment. This protein has mol. wt 94 000 and isoelectric point pH 4.8. This protein was also seen to increase after labeling liver slices with [^{35}S]methionine (unpublished results), although incorporation was too low to obtain completely satisfactory autoradiograms. The two-dimensional gel patterns of liver mitochondria matrix proteins published [26] do not appear to show the 94 000 mol. wt protein of p*K* 4.8. Hence this protein could be an outer membrane protein. A major carbohydrate-containing polypeptide with mol. wt ~93 000 has been detected [23] in outer membrane mitochondrial preparations. This protein could be the same, although we have no direct evidence on this point. Clearly to estimate protein synthetic rates the use of an isotopic label would be desirable. Work is in progress to obtain adequate incorporation of label for such studies.

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