Biochimica et Biophysica Acta 851 (1986) 49–56
Elsevier

BBA 42087

The induction of mitochondrial L-3-glycerophosphate dehydrogenase by thyroid hormone

Valerie J. Taylor and C. Ian Ragan

Department of Biochemistry, University of Southampton, Southampton, S09 3TU (U.K.)

(Received February 13th, 1986)

Key words: L-3-Glycerophosphate dehydrogenase; Thyroid hormone; Enzyme induction; (Porcine brain mitochondria)

L-3-Glycerophosphate dehydrogenase was purified from porcine brain mitochondria by a shorter and simpler procedure than previously reported. Immunoblotting with antiserum to the porcine enzyme established that rat liver L-3-glycerophosphate dehydrogenase has the same M_r (76000) by SDS-polyacrylamide gel electrophoresis. In liver mitochondria from normal and hyperthyroid rats, changes in L-3-glycerophosphate dehydrogenase activity were parallelled by changes in enzyme content assayed by immunoblotting. Similar changes were found in the amount of enzyme synthesised in vitro by reticulocyte lysate programmed with rat liver mRNA, suggesting that thyroid hormone causes specific induction of L-3-glycerophosphate dehydrogenase mRNA.

Introduction

Mitochondrial L-3-glycerophosphate dehydrogenase (EC 1.1.99.5) is a membrane-bound enzyme which catalyses the transfer of electrons from substrate to ubiquinone [1,3]. The enzyme has been purified from rabbit skeletal muscle and rabbit brain [3] and porcine brain [1] and, from all three sources, consists of a single polypeptide chain of M_r 76000 when analysed by SDS-polyacrylamide gel electrophoresis. The porcine brain enzyme reduces ubiquinone analogues and may be reconstituted with bovine heart ubiquinol-cytochrome *c* reductase and ubiquinone-10 to give L-3-glycerophosphate-cytochrome *c* oxidoreductase activity [2]. Unusually for a respiratory chain dehydrogenase, no firm evidence for the presence of iron-sulphur prosthetic groups has been obtained and it seems likely that the enzyme contains just 1 mol FAD per 76000 g protein. Both purification procedures rely on preparative isoelectric focussing which causes loss of activity and FAD, particularly from the porcine brain enzyme, thus preventing definite conclusions about the prosthetic group content [1].

The most likely role of the mammalian enzyme is in regulating the activity of the glycerophosphate 'shuttle' for the oxidation of cytoplasmic NADH [4]. Consistent with this is the regulation of the enzyme by Ca^{2+} [5] and thyroid hormone [6,7]. The latter effect is very tissue specific, the most dramatic effects occurring in liver where hyperthyroidism can lead to a 20-fold increase in activity [7]. Regulation of L-3-glycerophosphate dehydrogenase by thyroid hormone is more marked than with most other mitochondrial enzymes [8], and it is certainly the only respiratory-chain-linked enzyme to show a substantial response.

In this report, we describe a much simplified procedure for extensive purification of the enzyme based on an early method devised by Dawson and Thorne [9]. The homogeneous protein can then be obtained either by preparative isoelectric focus-

Abbreviation: DCIP, dichlorophenolindophenol.

sing or by preparative SDS-gel electrophoresis. We describe the production of a specific antibody to the porcine enzyme, its cross-reaction with the rat liver enzyme, and its use for studying the regulation of the latter enzyme by thyroid hormone.

Materials and Methods

Animals. Male Wistar rats (150 g) were made hyperthyroid by daily injections of 3,3′,5-triodo-L-thyronine (60 μg per animal) for 6 days.

Preparation of mitochondria. Porcine brain mitochondria were prepared from the cerebral hemispheres of up to 50 sows by the method of Cottingham and Ragan [1] adapted from that of Ringler [10]. For enzyme purification, the mitochondria were acetone dried and stored at −20°C in a vacuum desiccator over solid NaOH and paraffin wax. Rat liver mitochondria were prepared as described by Greenawalt [11] and submitochondrial particles according to Pedersen et al. [12]. These preparations were stored at −70°C in the presence of 1 μM phenylmethylsulphonylfluoride.

Purification of L-3-glycerophosphate dehydrogenase. All operations were carried out at 0–4°C. Acetone-dried mitochondria (12 g) were homogenised in 400 ml 30 mM potassium phosphate (pH 7.6) stirred for 20 min and centrifuged at 20000 × *g* for 15 min. The precipitate was then reextracted with the same vol. of buffer. The final precipitate was resuspended in 380 ml phosphate buffer to which was added 18 ml 6% (w/v) Triton X-100, and stirred for 20 min. After centrifugation at 100000 × *g* for 90 min, the supernatant was stirred for 1 h with hydroxylapatite. Prior to use, the hydroxylapatite (from BDH Chemicals or Bio-rad) was equilibrated with the phosphate buffer, washed with distilled water and allowed to settle under gravity. The excess water was discarded and 120 g of the packed material was used for the preparation. After centrifugation at 20000 × *g* for 3 min, the supernatant was concentrated to approx. 2 ml by ultrafiltration through an Amicon XM100 membrane under N_2 at 170 kPa. This procedure was adapted from that of Dawson and Thorne [9] and the enzyme at this state is referred to throughout as the 'partially purified enzyme'.

Further purification could be obtained either by preparative isoelectric focussing as described by Cottingham and Ragan [1] or by preparative SDS-gel electrophoresis as described by Cleeter et al. [13].

This procedure was also found to be suitable for the rat liver enzyme, which was purified through the hydroxylapatite and concentration stages from three rat livers.

Assays. L-3-glycerophosphate dehydrogenase was measured with ubiquinone-1 or DCIP as acceptors as described by Cottingham and Ragan [1]. Reconstitution of the enzyme with bovine heart Complex III, ubiquinone-10 and lipid, and assay with cytochrome *c* as acceptor was done according to Cottingham and Ragan [2]. Flavin was assayed by the method of Faeder and Siegel [14]. Protein was measured by the method of Lowry et al. [15] using fraction V bovine serum albumin (Sigma) as a standard.

SDS-polyacrylamide gel electrophoresis. This was carried out on 1.5 mm thick slab gels using the Laemmli [16] discontinuous buffer system. Protein bands were stained with brilliant blue R or electrophoretically transferred to nitrocellulose [17].

Preparation of antiserum. Purified L-3-glycerophosphate dehydrogenase from the isoelectric focussing step (200 μg of protein in 0.5 ml) was diluted with an equal volume of Freund's complete adjuvant (Miles Laboratories). A New Zealand white rabbit (4 kg) was immunised by multiple subcutaneous injections into the back. A booster, with the same amount of protein in Freund's incomplete adjuvant, was given 4 weeks later intramuscularly into the flank. Blood was collected from the marginal ear vein after 2 weeks and at fortnightly intervals after that. Serum was obtained by centrifugation of blood that had been left to clot at room temperature for 2 h.

Immunoprecipitation. Partially purified porcine brain enzyme (50 μg of protein) or rat liver enzyme (50 μg of protein) was incubated in a final volume of 0.5 ml containing 1% (w/v) Triton X-100, 30 mM potassium phosphate (pH 7.6), and various volumes of antiserum. Preimmune serum was also added to maintain the total volume of serum at 40 μl. After overnight incubation at 4°C, the samples were assayed for enzyme activity before and after centrifugation at 30000 × *g* for 30 min.

Immunoblotting. Nitrocellulose sheets were shaken overnight with antiserum diluted 100-fold with phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin [17]. Antibody-antigen complexes were decorated by sequential binding of biotinylated donkey anti-rabbit IgG (diluted 1:500) and streptavidin-biotinylated peroxidase complex (diluted 1:400) from Amersham International, and visualised using Biorad horse radish peroxidase reagent and H_2O_2. To lower the background colour, the nitrocellulose sheets were washed in phosphate-buffered saline containing 0.05% (w/v) Tween 20 after the antibody binding steps. All other washes and additions were made with phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin.

Preparation of total RNA and mRNA. Total cellular RNA was isolated from rat liver by the guanidine isothiocyanate method (Chirgwin et al. [18]). Poly (A^+) RNA was prepared from total RNA using messenger affinity paper (Hybond mAP, Amersham International plc) as described by Werner et al. [19].

In vitro translation. Translation was carried out in a mixture containing 12.8 μl of rabbit reticulocyte lysate (Amersham International plc), 1 μl of tRNA (1.4 mg/ml), 0.24 μl of placental ribonuclease inhibitor (Anglian Biotechnology Ltd.), 4 μl (30 μCi) of L-[^{35}Sl]-methionine (Amersham International plc), 1.46 μl of 2.6 mM magnesium acetate/1.08 M potassium acetate buffer and either 20 μg of total RNA or 1 μg of poly (A^+) RNA in 4 μl of water. The mixture was incubated at 30°C for 30 or 60 min.

Immuneadsorption of in vitro translated protein. L-3-Glycerophosphate dehydrogenase was immuneadsorbed from a 20 μl translation mixture onto fixed *Staphylococcus aureus* essentially as described by Anderson and Blobel [20], except that the dilution buffer contained, in addition, 3 mM *p*-aminobenzamidine and 0.2 mM each of leupeptin, antipain, chymostatin and pepstatin (Sigma Chemical Co.). Samples were incubated at 4°C for 4 h with 10 μl of preimmune serum and adsorbed with 100 μl of Pansorbin (Calbiochem) for 1 h at room temperature. The supernatant was incubated for 16 h with 10 μl of antiserum and adsorbed with Pansorbin as above. After washing [20], adsorbed protein was solubilised with 20 μl of 0.5 M Tris-HCl, (pH 6.8), 20 μl of 10% (w/v) SDS and 20 μl of 10% (v/v) 2-mercaptoethanol at 100°C for 2 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and analysed by fluorography using Amplify (Amersham International plc) as the fluorophore, and preflashed Kodak X-Omat AR film at −70°C.

Results

Purification of L-3-glycerophosphate dehydrogenase

Dawson and Thorne [9] found that the enzyme can be selectively extracted from acetone-dried porcine brain mitochondria with Triton X-100. Further purification could be achieved by adsorption of unwanted proteins onto hydroxylapatite (Table I) which leaves the enzyme considerably purified but very dilute (approx. 0.1 mg protein/ml). We have found that ultrafiltration through an Amicon XM100 membrane can give up to 100-fold increase in concentration with good retention of activity, while most of the Triton X-100 passes through. A small degree of purification also occurs during this step and SDS-gel electrophoresis of the concentrated enzyme (Fig. 1) reveals that the major component is a polypeptide of M_r 76000.

Purification to homogeneity was achieved either by preparative isoelectric focussing as described by Cottingham and Ragan [1] or by preparative SDS-gel electrophoresis (Fig. 1).

Enzymic properties of L-3-glycerophosphate dehydrogenase

The partially purified enzyme or enzyme from the isoelectric focussing step catalysed the reduction of DCIP or ubiquinone-1 L-3-glycerophosphate. Activities were comparable with those found previously for the porcine brain enzyme isolated by a different procedure (Table II). The partially purified enzyme could also be reconstituted with phospholipid, ubiquinone-10 and bovine heart ubiquinol-cytochrome *c* reductase to give antimycin-sensitive L-3-glycerophosphate-cytochrome *c* reductase [2].

The flavin content of the enzyme was also comparable to that of earlier preparations. Approx. 90% of the flavin was FAD, and contents were between 0.62–1.00 nmol/mg of protein for

TABLE I

PURIFICATION OF L-3-GLYCEROPHOSPHATE DEHYDROGENASE

Figures are taken from a single preparation. Values in parentheses show the range of specific activities obtained with seven different preparations. Activities were measured with DCIP as acceptor. The enzyme from the step 'after concentration' is the 'partially purified enzyme' described in the text.

Step	Volume (ml)	Activity (μmol/min per ml)	Protein concn. (mg/ml)	Specific activity (μmol/min per mg protein)	Purification (fold)	Yield of activity (%)
Acetone-dried mitochondria	400	0.127	7.5	0.0169 (0.0116–0.0169)	1	100
P_i-washed mitochondria	420	0.119	6.2	0.0192 (0.0149–0.0234)	1.1	98
Triton supernatant	380	0.125	0.78	0.160 (0.160 –0.285)	9.5	93
Hydroxylapatite supernatant	450	0.059	0.099	0.596 (0.596 –1.05)	35.3	52
After concentration	1.7	8.19	13.0	0.63 (0.63 –2.82)	37.3	27
After isoelectric focussing	2.1	0.847	0.66	1.28	75.7	3.5

the partially purified enzyme and 1.3 nmol/mg of protein for a preparation of the enzyme from the isoelectric focussing step. As discussed previously [1], the latter value is considerably less than would be expected for 1mol of FAD per 76000 g protein and is a result of the acidic isoelectric point of the enzyme. As shown in Table I, the yield of activity from this step is low and the increase in specific activity is much less than expected from the degree of protein purification (Fig. 1). This preparation is therefore still unsuitable for studies of enzyme structure and function. The rabbit enzyme survives isoelectric focussing rather better but is still rather FAD-deficient [3]. A purification procedure which avoids isoelectric focussing has been devised by Beleznai et al. [21] for the rat liver protein but again the flavin content is lower than would be expected and the specific-activity increase is not as great as the degree of protein

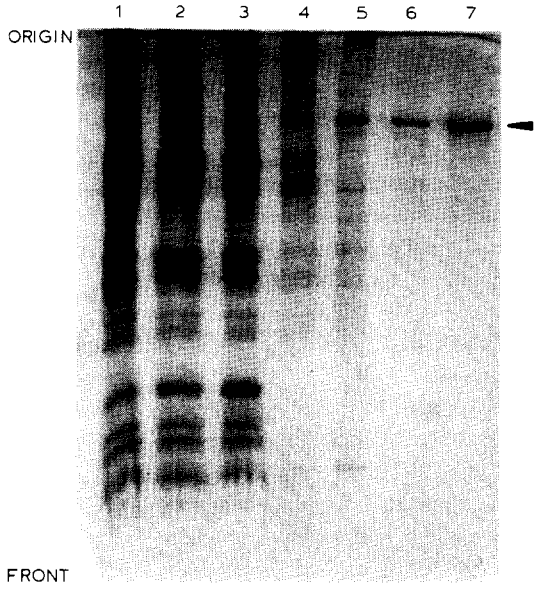

Fig. 1. Purification of L-3-glycerophosphate dehydrogenase. Samples from a purification were analysed on a 12–16% polyacrylamide gradient gel. Track 1, acetone-dried mitochondria (40 μg of protein); track 2, phosphate-washed mitochondria (40 μg of protein); track 3, Triton-treated mitochondria (40 μg of protein); track 4, supernatant from Triton extraction (24 μg of protein); track 5, enzyme after hydroxylapatite and concentration (10 μg of protein); track 6, enzyme after isoelectric focussing (4 μg of protein); track 7, protein from preparative SDS gel electrophoresis (4 μg of protein).

TABLE II

CATALYTIC PROPERTIES OF L-3-GLYCEROPHOSPHATE DEHYDROGENASE

Activities are for the 'partially purified' porcine brain enzyme. Cytochrome *c* was used as an acceptor after reconstitution with Complex III, ubiquinone-10 and phospholipid.

Acceptor	Specific activity (μmol/min per mg protein)		
	range	mean	S.E.
DCIP	0.63–2.82	1.52	0.21 ($n=11$)
Ubiquinone-1	1.31–4.38	3.15	0.65 ($n=4$)
Cytochrome *c*	4.46	4.46	($n=1$)

purification. However, the procedure described in the present paper is rapid and reproducible and provides ample pure protein for production of antibody.

Preparation of monospecific antiserum to L-3-glycerophosphate dehydrogenase

Homogeneous enzyme from the isoelectric focussing step was used as antigen. The resulting serum was effective in immuneprecipitating L-3-glycerophosphate dehydrogenase activity from solutions of the crude porcine brain enzyme, but caused little inhibition of activity (Fig. 2a). Using a partially purified preparation from rat liver, we could also demonstrate agglutination of the rat enzyme by the antiserum, again without loss of activity (Fig. 2b).

On immunoblots (Fig. 3) a single immunoreactive polypeptide of M_r 76000 was detected in the crude enzyme, in porcine brain mitochondria or in rat liver mitochondria. The specific activities of the porcine brain and rat liver mitochondria were very similar, and therefore the samples loaded onto the gel contained the same amount of enzyme activity. The similar intensities of the bands on the immunoblot therefore suggests that the porcine and rat enzymes showed similar reactivity towards the antibody.

At higher sensitivity on immunoblots of mitochondria or submitochondrial particles, other immunoreactive species could sometimes be detected. Products of M_r lower than 76000 were

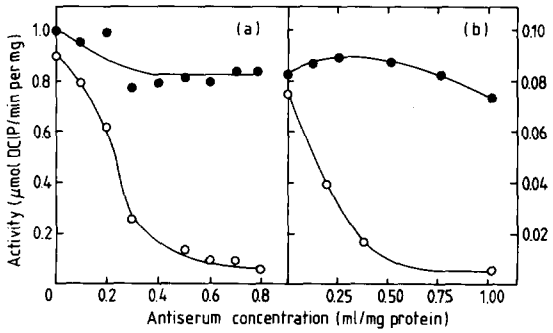

Fig. 2. Immuneprecipitation of L-3-glycerophosphate dehydrogenase. Immuneprecipitation was carried out as described in the Materials and Methods section using (a), partially purified porcine brain enzyme or (b), partially purified rat liver enzyme. Activity was measured before (●) and after (○) centrifugation.

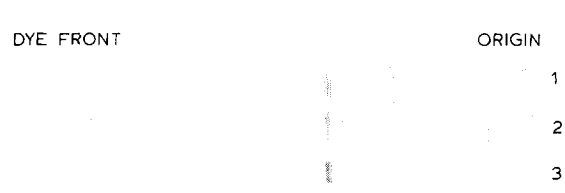

Fig. 3. Immunoblotting of L-3-glycerophosphate dehydrogenase. Samples were separated on a 10% polyacrylamide gel and transferred to nitrocellulose. Track 1, rat liver submitochondrial particles from hyperthyroid rats (100 μg protein); track 2, porcine brain submitochondrial particles (100 μg of protein); track 3, partially purified porcine enzyme (4 μg of protein).

more visible in stored material (particularly rat liver mitochondria) and could be minimised by storage at −70°C in the presence of 1 μM phenylmethylsulphonylfluoride, suggesting that they arose from proteolytic degradation. Two reactive species of higher M_r were often present and we considered that they might arise from a putative precursor form of the enzyme or a dimer. We could demonstrate that this was not the case by preabsorbing the antiserum with purified L-3-glycerophosphate dehydrogenase. The treated antiserum failed to react with the 76000 M_r species on immunoblots of mitochondria, but still visualised the higher M_r species. The latter therefore were immunologically distinct from L-3-glycerophosphate dehydrogenase.

The effect of thyroid status on the level of L-3-glycerophosphate dehydrogenase

In agreement with other workers [6,7], we found that the L-3-glycerophosphate dehydrogenase activity of rat liver mitochondria was markedly dependent on the thyroid status of the animal. In mitochondria from hyperthyroid animals, the activity of 15.7 ± 2.9 S.E. (n = 19) nmol/min per mg of mitochondrial protein was, on average, 6.5-fold higher than in mitochondria from untreated rats (2.4 ± 0.4 S.E. (n = 8) nmol/min per mg of protein). The activity changes were parallelled by changes in the amount of enzyme detected by immunoblotting. Fig. 4a shows the results obtained with liver mitochondria from normal and hyperthyroid rats. The intensity of the 76000 M_r protein, as determined by densitometry, was approx. 6-fold higher in the hyperthyroid state com-

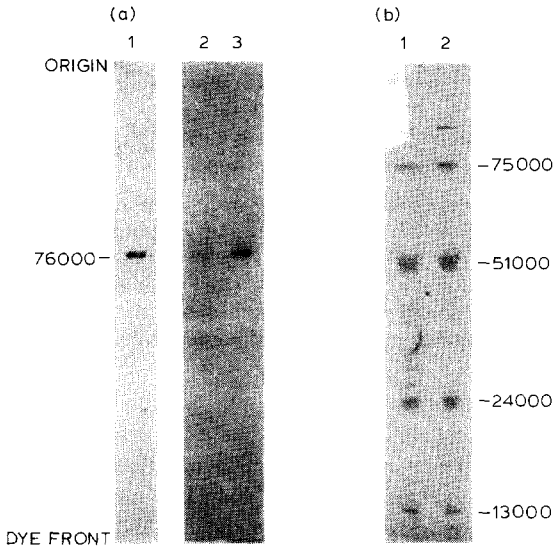

Fig. 4. Effect of thyroid hormone on the mitochondrial contents of L-3-glycerophosphate and NADH dehydrogenase. Immunoblotting was carried out on nitrocellulose transfers from a 10% polyacrylamide (a) or a 12% polyacrylamide gel (b). In (a) blotting was with antiserum to L-3-glycerophosphate dehydrogenase; track 1, partially purified porcine enzyme (4 μg protein); track 2, liver submitochondrial particles from normal rat (100 μg protein); track 3, liver submitochondrial particles from a hyperthyroid rat (100 μg of protein). In (b), blotting was with antiserum to bovine heart Complex I; track 1, as track 3 in (a); track 2, as track 2 in (a). Molecular masses of major subunits are indicated.

pared with normal. The activities of the two preparations were, respectively, 18.2 and 2.2 nmol/min per mg protein (i.e., 8-fold higher in the hyperthyroid state).

That the effect of thyroid hormone on the amount of L-3-glycerophosphate dehydrogenase protein shows specificity is demonstrated in Fig. 4b. In the hyperthyroid state, NADH dehydrogenase activity is not elevated, and immunoblotting of rat liver proteins with antiserum to bovine heart Complex I (Cleeter and Ragan [22]) reveals no effect of thyroid status on the levels of NADH dehydrogenase polypeptides.

In vitro translation of rat liver mRNA

To investigate whether the elevation of L-3-glycerophosphate dehydrogenase activity and protein by thyroid hormone was due to an elevated level of mRNA, we have used in vitro translation as an indirect assay of mRNA levels.

In the experiment of Fig. 5a, reticulocyte lysate was programmed with total rat liver RNA such that the incorporation of ^{35}S-methionine into total protein was the same whether the RNA was derived from normal or from hyperthyroid animals. The level of L-3-glycerophosphate dehydrogenase protein produced was, however, several-fold higher with RNA from hyperthyroid rats. The same effect was found when purified mRNA was used (Fig. 5b). In this experiment, the amounts of mRNA were chosen to be suboptimal for translation so that the incorporation of ^{35}S-methionine into protein was proportional to mRNA concentration. Total count incorporation was the same for mRNA from normal and hyperthyroid animals, but the latter produced much higher levels of incorporation into L-3-glycerophosphate dehydrogenase.

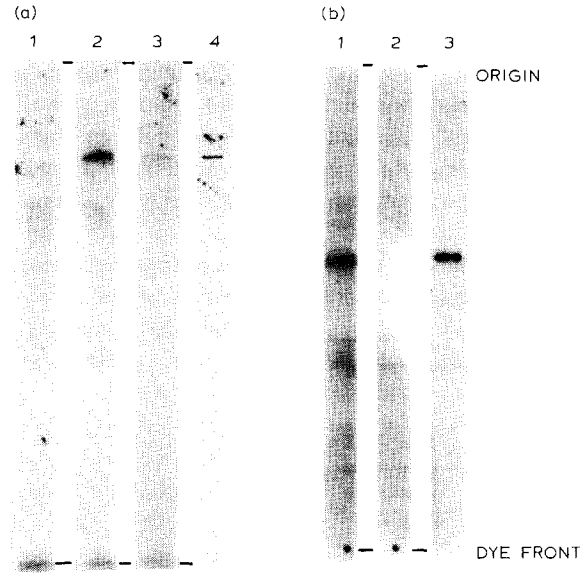

Fig. 5. In vitro translation of L-3-glycerophosphate dehydrogenase. In (a), the translation was carried out using total rat liver RNA fractions and products were analysed on a 12% polyacrylamide gel. Track 1, control with no antiserum present; track 2, translation with RNA from a hyperthyroid rat; track 3, translation with RNA from a normal rat; track 4, position of porcine brain L-3-glycerophosphate dehydrogenase. The protein was located by staining of the gel and marked with radioactive ink. In (b), translation was carried out using pure poly (A^+) RNA fractions and products were analysed on a 10% polyacryamide gel. Track 1, as track 2 in (a); track 2, as track 3 in (a); track 3, as track 4 in (a).

These results demonstrate that thyroid hormone causes a specific increase in the level of mRNA for L-3-glycerophosphate dehydrogenase.

The apparent M_r of the protein translated in vitro is indistinguishable from that of the mature protein and we have checked this on gels of widely varying polyacrylamide concentrations. Thus we have, at this stage, no evidence for a larger precursor form of the protein.

Discussion

We have demonstrated that thyroid hormone induces in liver a large increase in L-3-glycerophosphate dehydrogenase protein commensurate with the increase in the mitochondrial activity of this enzyme. From the in vitro translation experiments, we suggest that this effect is due to stimulation of the rate of synthesis of the specific mRNA although we cannot exclude the possibility that the increase in mRNA level is a result of a decreased rate of degradation. As judged by immunoblotting, there seems to be little or no effect of thyroid hormone on the levels of NADH dehydrogenase proteins.

Studies by Nelson and co-workers [8,23] had demonstrated that thyroid hormone induces a generalised increase in those proteins translated on mitochondrial ribosomes and this was confirmed for several components of the respiratory chain complexes, but not for any constituents of NADH dehydrogenase. Cytoplasmically synthesised components of Complex III, the F_1-ATPase and cytochrome *c* oxidase also increased to a modest extent, but the effect was very much less marked than we find for L-3-glycerophosphate dehydrogenase, and it appears from our own work that proteins of the NADH dehydrogenase are not induced.

Joste et al. [24] found no evidence for a generalised increase in polysome content in hyperthyroid animals or in the translational activity of this mRNA. Our own work confirms the latter finding, but Joste et al. [24] found that there was not necessarily a correlation between the increased level of a specific mitochondrial membrane protein and the concentration of its mRNA. For example, in vitro translation of mRNA from hyperthyroid animals revealed increased levels of mRNAs specifying cytochrome c_1 and the Rieske iron-sulphur protein. However, mRNAs for the β subunit of F_1 and the core protein I of Complex III were not so induced despite the increased levels of these proteins in the mitochondria. For L-3-glycerophosphate dehydrogenase, there is a good correlation between the mRNA level and the induction of proteins, and our results are very similar to those obtained by Dozin et al. [25] for cytoplasmic malic enzyme, an enzyme which responds to thyroid hormone to the same large extent as L-3-glycerophosphate dehydrogenase. The more complicated results for the mitochondrial complexes may be a reflection of their assembly, requiring polypeptides derived from both cytoplasmic and mitochondrial ribosomes. The level of any particular protein in the membrane will not therefore be simply related to the mRNA content but will also depend on the availability of other constituent proteins of the complex.

Acknowledgements

This work was supported by a research grant from the Science and Engineering Research Council.

References

1 Cottingham, I.R. and Ragan, C.I. (1980) Biochem. J. 192, 9–18
2 Cottingham, I.R. and Ragan, C.I. (1980) Biochem. J.192, 19–31
3 Cole, E.S., Lepp, C.A., Holoan, P.D. and Fondy, T.P. (1978) J. Biol. Chem. 253, 7952–7959
4 Werner, H.V. and Berry, M.N. (1974) Eur. J. Biochem. 42, 315–324
5 Wernette, M.E., Ochs, R.S. and Lardy, H.A. (1981) J. Biol. Chem. 256, 12767–12771
6 Lee, Y.-P., Takemori, A.E. and Lardy, H.A. (1959) J. Biol. Chem. 234, 3051–3054
7 Lee, Y.-P. and Lardy, H.A. (1965) J. Biol. Chem. 240, 1427–1436
8 Mulvei, A., Göransson, D., Latruffe, N. and Nelson, B.D. (1985) Achievements and Perspectives of Mitochondrial Research (Quagliariello, E., Slater, E.C., Palmieri, F., Saccone, C. and Kroon, A.M., eds.), p. 168, Elsevier, Amsterdam
9 Dawson, A.P. and Thorne, C.J.R. (1969) Biochem. J. 114, 35–40
10 Ringler, R.L. (1961) J. Biol. Chem. 236, 1192–1198
11 Greenawalt, J.W. (1974) Methods Enzymol. 31, 315–316
12 Pedersen, P.L., Greenawalt, J.W., Reynafarje, B., Hulliken,

J., Decker, G.l., Soper, J.W. and Bustamente, E. (1978). Methods Cell Biol. 20, 450–456
13 Cleeter, M.J.W., Banister, S.H. and Ragan, C.I. (1985) Biochem. J. 227, 467–474
14 Faeder, E.J. and Siegel, L.M. (1973) Anal. Biochem. 53, 332–336
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Laemmli, U.K. (1970) Nature (London) 227, 680–685
17 Erickson, P.F., Minier, L.N., Lasher, R.S. (1981) J. Immun. Methods 51, 241–249
18 Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299
19 Werner, D., Chemla, Y. and Herzberg, M. (1984) Anal. Biochem. 141, 329–336
20 Anderson, D.J. and Blobel, G. Methods Enzymol. 96, 111–120
21 Beleznai, Zs. and Jancsik, V. (1985) Achievements and Perspectives of Mitochondrial Research, (Quagliariello, E., Slater, E.C., Palmieri, F., Saccone, C. and Kroon, A.M., eds.), p. 54, Elsevier, Amsterdam
22 Cleeter, M.J.W. and Ragan, C.I. (1985) Biochem. J. 230, 739–746
23 Nelson, B.D., Mutvei, A. and Joste, V. (1984) Arch. Biochem. Biophys. 228, 41–48
24 Joste, V., Nelson, B.D. and Kuzela, S. (1985) Achievements and Perspectives of Mitochondrial Research, (Quagliariello, E., Slatex, E.C., Palmieri, F., Saccone, C. and Kroon, A.M., eds.), p. 298, Elsevier, Amsterdam
25 Dozin, B., Magnuson, M.A. and Nikodem, V.M. (1985). Biochemistry 24, 5581–5586