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Mitochondrial metabolism in different thyroid states

Sibylle SOBOLL,*§ Claus HORST,† Holger HUMMERICH,‡ Jens P. SCHUMACHER* and Hans J. SEITZ†

*Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstrasse 5, Düsseldorf, †Institut für Physiologische Chemie, Universität Hamburg, Martinistrasse 52, Hamburg, and ‡Institut für Humangenetik, Universität Düsseldorf, Düsseldorf, Federal Republic of Germany

The protonmotive force, as well as the mitochondrial and cytosolic concentrations of malate, 2-oxoglutarate, glutamate and aspartate, were determined in livers from hypo-, eu- and hyper-thyroid rats, by density-gradient centrifugation of freeze-clamped livers in non-aqueous solvents [Soboll, Akerboom, Schwenke, Haase & Sies (1980) Biochem. J. **192**, 951–954]. The mitochondrial/cytosolic pH difference and the membrane potential were significantly enhanced in hyperthyroid livers compared with the hypothyroid state, resulting in an increased protonmotive force in the presence of thyroid hormones [Soboll & Sies (1989) Methods Enzymol. **174**, 118–130]. The mitochondrial concentrations of 2-oxoglutarate, glutamate and aspartate were significantly higher in the euthyroid than in the hypothyroid state, but only slightly higher in the hyperthyroid state. Mitochondrial malate, on the other hand, increased significantly from the hypothyroid to the hyperthyroid state. The mitochondrial/cytosolic concentration gradients were significantly increased in the presence of thyroid hormones only for malate. The changes in steady-state metabolite concentrations reflect a higher substrate supply and a stimulation of mitochondrial metabolism. However, a clear relationship between the increased protonmotive force, as the driving force for mitochondrial metabolite transport, and the subcellular metabolite concentrations is not observable in different thyroid states.

INTRODUCTION

Thyroid hormones stimulate respiration and gluconeogenesis in liver similarly to glucagon and α-adrenergic agonists (for a review see Soboll & Sies, 1989). Interestingly, for thyroid hormones these effects are observed within minutes of L-triiodothyronine application (Müller & Seitz, 1980), as well as in the long term (Müller & Seitz, 1981). Long-term effects appear to be due to enzyme induction: thus the capacity of malic enzyme (Oppenheimer, 1983), mitochondrial α-glycerophosphate dehydrogenase (Lee & Lardy, 1965), succinate dehydrogenase (Maddaiah *et al.*, 1981) and the cytochromes of the respiratory chain (Horrum *et al.*, 1985) is increased in hyperthyroid-rat livers.

The question of the mechanism of the stimulation of respiration and gluconeogenesis is still a matter of debate. Increased substrate supply to the respiratory chain is a prerequisite for the glucogenic action. Several studies suggest a stimulation of metabolite transport across the mitochondrial membrane in hyperthyroidism. (i) The $V_{max.}$ of the tricarboxylate carrier in hepatic mitochondria is increased (Paradies & Ruggiero, 1990), which was attributed to changes in the lipid composition of the inner membrane altering its fluidity. (ii) It has been reported that thyroid hormones stimulate adenine nucleotide transport in isolated mitochondria and in perfused liver (Babior *et al.*, 1973; Hoch, 1977; Palacios-Romero & Mowbray, 1979; Seitz *et al.*, 1985). However, gene expression for adenine nucleotide translocase was not enhanced in hyperthyroid-rat livers (Höppner *et al.*, 1988).

The stimulation of mitochondrial transport systems may be of a more indirect nature, e.g. by changing the driving forces for membrane transport, the mitochondrial/cytosolic proton gradient and the membrane potential (for a review see La Noue & Schoolwerth, 1979): thus glucagon, adrenaline and L-triiodothyronine within minutes increase the mitochondrial/cytosolic pH difference, ΔpH_m, in perfused liver (Soboll & Sies, 1989). Also, a stimulation of adenine nucleotide transport and of pyruvate transport has been observed with α-adrenergic agonists (Titheradge & Coore, 1976; Leverve *et al.*, 1986) and with glucagon (Halestrap & Armston, 1984). Both observations would be consistent with a more indirect stimulation of mitochondrial metabolite transport, although a direct effect on carrier proteins (e.g. protein phosphorylation) cannot be excluded. However, until now phosphorylation of mitochondrial carriers by glucagon or α-adrenergic agonists has not been observed.

In our study, we examined whether the thyroid state influences the driving forces for mitochondrial metabolite transport in rat liver *in vivo* and whether changes in the steady-state concentrations of metabolites may reflect a stimulation of metabolite transport. Mitochondrial and cytosolic metabolite concentrations were determined by fractionation of freeze-clamped rat liver in non-aqueous solvents (Soboll *et al.*, 1980*a*).

MATERIALS AND METHODS

Rat livers *in vivo*

In male Wistar rats (180–200 g) hypothyroidism was induced by intraperitoneal injection of $Na^{131}I$ (250 μCi/100 g body wt.) 21–28 days before the experiment. Hyperthyroidism was produced by daily intraperitoneal injections of thyroxine (50 μg/100 g body wt. for 7 days). The hypo- and hyper-thyroid states were monitored by serum thyroxine (< 10 and > 250 ng/ml of serum respectively). Experiments were performed in 48 h-fasted rats. Rapid liver sampling was performed on unanaesthetized unrestrained rats by the double-hatchet method (Faupel *et al.*, 1972).

Determination of the mitochondrial proton gradient and membrane potential

For determination of the mitochondrial proton gradient, rats were injected with 15 μCi of [2-^{14}C]5,5-dimethyl-2,4-oxazolidinedione ([^{14}C]DMO) 15 min before extraction of the

Abbreviations used: DMO, 5,5-dimethyl-2,4-oxazolidinedione; TPMP, triphenylmethylphosphonium bromide; ΔpH_m, mitochondrial/cytosolic pH difference; ψ_m, mitochondrial membrane potential; Δp_m, protonmotive force.

§ To whom correspondence should be addressed.

liver by the double-hatchet method. Blood samples were taken during killing of the animals for determination of the specific radioactivities of DMO in the extracellular space.

For determination of the mitochondrial membrane potential (ψ_m), rat livers were perfused in a closed system with 200 ml of Krebs–Henseleit bicarbonate buffer supplemented with 1 mM-pyruvate, 10 mM-L-lactate and 15 μCi of [^{14}C]triphenylmethylphosphonium bromide ([^{14}C]TPMP), under O_2/CO_2 (19:1), at pH 7.4 and 37 °C, for 30 min, and perfusate samples were taken for determination of specific radioactivities of TPMP in the extracellular space. Then the liver was freeze-clamped. Intraperitoneal injection of the label, as for DMO, was not possible, owing to the toxicity of TPMP.

Mitochondrial proton gradients and membrane potentials were calculated after determination of mitochondrial (m) and cytosolic (c) specific radioactivities of [^{14}C]DMO and [^{14}C]TPMP respectively, by fractionation in non-aqueous solvents as described below, according to eqns. (1) and (2):

$$\Delta pH_m = \log \frac{[[^{14}C]DMO]_m}{[[^{14}C]DMO]_c} \quad (1)$$

$$\psi_m = \frac{2.303\,RT}{n} \times F \log \frac{[[^{14}C]TPMP]_m}{[[^{14}C]TPMP]_c} \quad (2)$$

where n is no. of electrons and F is Faraday constant.

Fractionation of liver tissue in non-aqueous solvents

For the determination of mitochondrial and cytosolic contents of metabolites as well as specific radioactivities of DMO and TPMP, respectively, freeze-clamped rat livers were ground in liquid nitrogen, freeze-dried and sonicated in a mixture of heptane/CCl_4 at −10 °C and then fractionated by density-gradient centrifugation in a gradient obtained from heptane/CCl_4 mixtures (1.28–1.38 kg/l) in accordance with Soboll *et al.* (1980*a*). The gradient yielded eight fractions each containing different proportions of mitochondrial and cytosolic protein. Specific activities of marker enzymes for mitochondria (citrate synthase) and cytosol (phosphoglycerate kinase) and metabolite contents were measured enzymically (Bergmeyer, 1974; Soboll *et al.*, 1980*a*) in each fraction, and specific radioactivities were determined by liquid-scintillation counting. Protein contents were measured as described by Lowry *et al.* (1951). Mitochondrial and cytosolic metabolite contents and specific radioactivities were extrapolated from the activities of marker enzymes and the contents of metabolites in each fraction of the density gradient (Elbers *et al.*, 1974). Concentrations were calculated on the basis of mitochondrial and cytosolic water contents of 0.8 and 3.8 μl/mg of compartmental protein respectively (Soboll *et al.*, 1976). Corrections were made for extracellular specific radioactivities of [^{14}C]DMO and [^{14}C]TPMP in each fraction.

Materials

All enzymes and coenzymes were from either Boehringer (Mannheim, Germany) or Sigma (Munich, Germany). Chemicals were from Merck (Darmstadt, Germany). Radiochemicals were from Amersham Buchler (Braunschweig, Germany) and NEN (Dreieichen, Germany).

RESULTS AND DISCUSSION

In the euthyroid state the mitochondrial concentrations of malate, 2-oxoglutarate, glutamate and aspartate are increased compared with the hypothyroid state (Table 1). These effects are partially reversed in the hyperthyroid state, except for malate, which is now significantly elevated. These changes are also reflected in the mitochondrial/cytosolic concentration ratios (Table 2), which are an estimate for the driving forces of mitochondrial metabolite transport. The mitochondrial/cytosolic concentration ratios of 2-oxoglutarate and malate increase from hypo- to eu- to hyper-thyroid-rat livers, whereas the ratios from aspartate and glutamate increase from the hypo- to the eu-thyroid state, but decrease from the euthyroid to the hyperthyroid state.

Several transport systems located in the mitochondrial inner membrane are proton-compensated, such as the transport of di- and tri-carboxylic acids and phosphate, whereas the glutamate/aspartate as well as the adenine nucleotide carrier are electrogenic and therefore dependent on the mitochondrial membrane potential (La Noue & Schoolwerth, 1979). As shown in Table 2, both $\Delta\psi$ and ΔpH are increased in the presence of thyroid hormone and are significantly elevated in the hyperthyroid compared with the hypothyroid state. For malate and 2-oxoglutarate, the steady-state concentration ratios reflect the

Table 1. Subcellular distribution of metabolites in rat liver *in vivo* under different thyroid states

Results are means ± S.E.M. ($n = 4$): $^*P < 0.05$, or †not significant, versus hypothyroid state.

	Metabolite concn. (mM)		
	Hypothyroid	Euthyroid	Hyperthyroid
Mitochondria			
2-Oxoglutarate	0.68 ± 0.16	1.56 ± 0.08*	0.88 ± 0.25†
Malate	0.93 ± 0.12	1.18 ± 0.14†	1.38 ± 0.16*
Aspartate	1.74 ± 0.25	2.99 ± 0.15*	1.99 ± 0.23†
Glutamate	5.90 ± 0.75	10.6 ± 1.5*	7.85 ± 0.93†
Cytosol			
2-Oxoglutarate	0.08 ± 0.02	0.12 ± 0.01	0.07 ± 0.01
Malate	0.40 ± 0.13	0.24 ± 0.02	0.24 ± 0.04
Aspartate	0.46 ± 0.13	0.55 ± 0.06	0.68 ± 0.09
Glutamate	2.95 ± 0.47	3.10 ± 0.32	3.03 ± 0.46

Table 2. Subcellular concentration ratios and protonmotive force in rat livers *in vivo* in different thyroid states

Results are means ± S.E.M. ($n = 4$); the concentration ratios were calculated from data from Table 1. The membrane potential ψ_m was calculated from the mitochondrial/cytosolic distribution of [^{14}C]TPMP, and ΔpH_m from the distribution of [^{14}C]DMO. $^*P < 0.05$, or †not significant, versus hypothyroid state. $\Delta p_m = \psi_m + 61\Delta pH_m$.

	Hypothyroid	Euthyroid	Hyperthyroid
Mitochondrial/cytosolic gradient			
2-Oxoglutarate	11.1 ± 5.0	13.3 ± 1.7†	21.0 ± 12†
Malate	2.9 ± 0.8	5.0 ± 0.5*	6.5 ± 1.5*
Aspartate	4.5 ± 1.0	5.7 ± 0.7†	3.1 ± 0.5†
Glutamate	2.0 ± 0.2	3.4 ± 0.4*	2.8 ± 0.5†
ΔpH_m calculated from the distribution of:			
DMO	0.39 ± 0.09	0.54 ± 0.04*	0.66 ± 0.08*
2-Oxoglutarate	0.49 ± 0.08	0.56 ± 0.03†	0.57 ± 0.13†
Malate	0.21 ± 0.06	0.35 ± 0.02*	0.42 ± 0.04*
Glutamate	0.30 ± 0.04	0.53 ± 0.05*	0.45 ± 0.08†
ψ_m (mV)	134 ± 7	141 ± 2	149 ± 3*
Δp_m (mV)	161 ± 11	175 ± 4	192 ± 5*

changes in mitochondrial/cytosolic proton gradients in different thyroid states, although they do not fully equilibrate and although the changes are not significant with 2-oxoglutarate. However, especially in hyperthyroidism, metabolic rates, such as fatty acid synthesis (Landriscina *et al.*, 1976), gluconeogenesis, respiration and urea synthesis (Müller & Seitz, 1981), are increased, and the metabolic drain in biosynthetic pathways may counterbalance the effect of the stimulation of metabolite transport on the mitochondrial metabolite concentrations. For example, whereas in the euthyroid state the mitochondrial concentrations of 2-oxoglutarate, glutamate and aspartate are elevated compared with the hypothyroid state, they reverse to close to the hypothyroid values in hyperthyroidism.

On the other hand, the distribution ratio for glutamate/aspartate exchange is far from equilibrium with the membrane potential, whereas it was shown previously that in hyperthyroid rats the changes in mitochondrial and cytosolic ATP and ADP, which are also transported electrogenically, are consistent with an increase in the mitochondrial membrane potential (Seitz *et al.*, 1985). This may have several reasons. Firstly, the mitochondrial concentration of aspartate is below the K_m value for the glutamate/aspartate carrier (3 mM for internal aspartate; La Noue & Schoolwerth, 1979); an equilibration of aspartate with the mitochondrial membrane potential (mitochondria/cytosol ratio for aspartate of approx. 0.01) would decrease mitochondrial aspartate to the micromolar range. Therefore the translocator will hardly be able to establish distribution equilibrium. Indeed, in earlier measurements in perfused liver, as well as in isolated hepatocytes under different metabolic conditions, mitochondrial/cytosolic concentration gradients not lower than 0.5 (Soboll *et al.*, 1976; Siess *et al.*, 1977) were measured for aspartate. Secondly, during increased gluconeogenesis and urea synthesis in hyperthyroid starved rats, cytosolic aspartate is used at high rates as precursor for oxaloacetate and argininosuccinate, respectively, opposing an accumulation of aspartate in the cytosol. Thirdly, glutamate is additionally transported by another glutamate/proton symport system, and indeed the distribution of glutamate follows the proton gradient rather than the membrane potential (Table 2). Similar observations have been made earlier in livers from fed and fasted rats (Soboll *et al.*, 1980*b*).

Thyroid hormones increase the mitochondrial proton gradient as well as the membrane potential significantly from the hypo- to the hyper-thyroid state (Table 2). The increased protonmotive force indicates a higher energization of the mitochondria in the presence of thyroid hormones, which is in line with the finding of an increase in glucose output under the same conditions (Müller & Seitz, 1981). An increase in the mitochondrial membrane potential in the presence of thyroid hormones would also explain the observation of a higher rate of adenine nucleotide transport in mitochondria from euthyroid-rat livers than in those from hypothyroid-rat livers (Hoch, 1977; Seitz *et al.*, 1985).

A high protonmotive force also increases the proton conductance of the mitochondrial membrane. An increased proton leak in hepatocytes from hyperthyroid rats was consistently found by Brand (1990) and is supposed to be responsible for increased heat production in hyperthyroidism.

Glucagon and adrenaline, which are known to stimulate respiration and gluconeogenesis in liver, like thyroid hormone, also increase ΔpH_m (Soboll & Scholz, 1986). However, only a minor change in the mitochondrial membrane potential in the presence of glucagon has been demonstrated in isolated hepatocytes (Strzelecki *et al.*, 1988). Therefore it is still open whether these different hormones stimulate mitochondrial metabolism by similar mechanisms. For thyroid hormones, changes in Ca^{2+} fluxes (Shears & Bronk, 1979) or the increased capacity of the respiratory chain could be responsible for the change in the steady-state protonmotive force.

Summarizing, the stimulatory action of thyroid hormones on mitochondrial metabolism appears to be the concerted action on enzymic capacity, i.e. increase in respiratory-chain complexes and enzyme activities, and an increase in the driving forces for mitochondrial metabolite transport via an elevation of the mitochondrial protonmotive force. However, a clear correlation between the subcellular distribution of key metabolites in mitochondrial metabolism and the respective driving forces for their transport systems is not found in intact rat liver.

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