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Effect of Chemical Modification *In Situ* on L-Glycerol-3-Phosphate Dehydrogenase in Brown Adipose Tissue Mitochondria

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Abstract

Mitochondrial L-glycerol-3-phosphate dehydrogenase (E.C. 1.1.99.5.) was studied by chemical modification *in situ* with different amino acid side chain specific reagents in mitochondria isolated from hamster brown adipose tissue. The SH-modifying reagents have only slight effect on the enzyme activity. The most effective chemicals were tetranitromethane and diazobenzene sulfonic acid. The enzyme activity can be abolished completely by both of them. In the presence of Ca^{2+} and/or glycerol-3-phosphate inhibition was greater at the same electrophilic reagent concentration. The effect of Ca^{2+} and glycerol-3-phosphate is nonadditive on inhibition by these reagents.

Key Words: Mitochondrial L-glycerol-3-phosphate dehydrogenase; brown adipose tissue; chemical modification; tetranitromethane; diazobenzene sulfonic acid.

Introduction

Cytosolic NADH-dependent glycerol-3-phosphate dehydrogenase (EC. 1.1.8.1.) together with mitochondrial inner membrane-bound FAD-linked L-glycerol-3-phosphate dehydrogenase (EC. 1.1.99.5.) forms the glycerol-3-phosphate shuttle, which transports the reducing equivalents into the mitochondria. Through this mechanism, the cytosolic NAD^+/NADH ratio is effectively controlled by the mitochondrial respiratory chain enzymes.

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The shuttle was first described in insect flight muscle (Zebe *et al.*, 1957). There are also other tissues like kidney, placenta, or pancreatic islets where this shuttle system can play an important regulatory role (Dawson and Cooney, 1978; Swierczynski *et al.*, 1976; MacDonald, 1981). The glycerol-3-phosphate shuttle was also proposed in brown adipose tissue by Okhawa *et al.* (1969). In this tissue both enzymes have high activity, which lends high efficiency to the shuttle function. Brown adipose tissue plays an important role in nonshivering thermogenesis in new-born mammals and adult hibernating animals. Therefore, in this tissue the glycerol-3-phospate shuttle is involved in the mechanism that regulates heat production. The reaction catalyzed by mitochondrial L-glycerol-3-phosphate dehydrogenase is the rate-limiting step in the glycerol-3-phosphate shuttle. Therefore, the key regulatory enzyme of the shuttle system is the mitochondrial one (Werner and Berry, 1973; Pösö, 1977). Its activity can be modulated over a wide range by various cytosolic factors like Ca^{2+} (Estabrook and Sacktor, 1958; Carafoli and Sacktor, 1972; Wernette *et al.*, 1981), acyl-CoA esters (Bukowiecki and Lindberg, 1974), or free fatty acids (Rauchová and Drahota, 1984). The mechanisms through which various ligands regulate the mitochondrial L-glycerol-3-phosphate dehydrogenase action are not fully understood. They might act *via* binding to different parts of the protein molecule inducing conformational changes. By this mechanism the properties of catalytic sites, as well as binding sites for cofactor, substrate, and hydrogen acceptor(s), may be changed. This is supported by various findings on purified L-glycerol-3-phosphate dehydrogenase or *in situ* mitochondria showing that the enzyme has functionally important SH-groups (Dawson and Thorne, 1975; Rauchová *et al.*, 1985; Shi Shang Ping *et al.*, 1986; Garrib and McMurray, 1986). Recent observations indicate that cysteinyl side chains are required for Ca^{2+} and Mg^{2+} binding to the enzyme molecule (Beleznai *et al.*, 1988).

In the present work we analyzed by chemical modifications the role of some amino acid side chains of L-glycerol-3-phosphate dehydrogenase on its function *in situ*. Our results indicate that reagents reacting preferentially with tyrosyl, lysyl, or histidyl side chains are inhibitors of the L-glycerol-3-phosphate dehydrogenase activity in mitochondria isolated from brown adipose tissue.

Materials and Methods

Chemicals

Tetranitromethane (TNM) and phenylglyoxal monohydrate (PGO) were obtained from Aldrich Europe (Beerse, Belgium), 2,4,6-trinitrobenzene

sulphonic acid (TNBS) from VU Rybitví (Pardubice, Czechoslovakia), *N,N'*-dicyclohexyl carbodiimide (DCCD), diethyl pyrocarbonate (DEPC), mersalyl, *p*-chloromercuribenzoate, *N*-ethylmaleimide, *N,N'*-phenylenedimaleimides (ortho and para derivatives), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) from Sigma Chemical Co. (St. Louis, Missouri), eosin from Merck, and eosin-5-maleimide from Molecular Probes Inc., (USA). All other chemicals were of analytical grade from Sigma Chemicals Co. (St. Louis, Missouri).

Preparation of Mitochondria

The experiments were performed on mitochondria isolated from interscapular, cervical, and axillary brown adipose tissue of adult male hamsters (*Mesocricetus auratus*) that had been cold adapted at 4° C for at least two weeks and had food and water *ad libitum*. Mitochondria were prepared as described previously (Hittelman *et al.*, 1969). The isolated mitochondria were further washed and finally resuspended in 0.25 mol/l sucrose, 1 mmol/l EDTA, and 10 mmol/l potassium phosphate, pH = 7.4.

Preparation of Protein-Modifying Reagents and Treatment of Mitochondria

Protein-modifying reagents were prepared as follows: diazobenzene sulfonic acid (DABS) according to Tinberg *et al.* (1974), DTNB according to Aquila and Klingenberg (1982), *N*-ethylmaleimide according to Fonyó and Vignais (1980), PGO according to Werber *et al.*, (1975), TNM according to Guerrieri and Papa (1981), DEPC according to Ovádi *et al.* (1967) and Guerrieri and Papa (1981).

The reaction with TNBS was performed at 20°C in 0.25 mol/l sucrose and 20 mmol/l morpholino-propanesulfonic acid (MOPS), pH = 8.1, and was stopped by the addition of 7 mmol/l HCl to get pH 6.6.

DCCD was dissolved in ethanol at 500 mmol/l concentration.

As SH-group modifying reagents, 25 mmol/l mersalyl and *p*-chloromercuribenzoate (pH adjusted to 7.0) were used.

Eosin-5-maleimide, *N,N'-o*-phenylenedimaleimide and *N,N'-p*-phenylenedimaleimide were dissolved in dimethylsulphoxide.

The titration curves were fitted and linearized with a Hewlett-Packard 85 calculator. It was used for the calculation of the 50% inhibition values.

Data shown in the titration curves are the end-points of the chemical modifying reaction.

Determination of Mitochondrial L-Glycerol-3-Phosphate Dehydrogenase Activity

The activity of mitochondrial L-glycerol-3-phosphate dehydrogenase was determined at 25°C using a Clark-type oxygen electrode (Estabrook,

1967) in a medium containing 0.25 mol/l sucrose, 1 mmol/l EDTA, 5 mmol/l $CaCl_2$, 1 mmol/l KCN, and 3 mmol/l phenazinemethosulphate, 50 mmol/l sn-glycerol-3-phosphate, 10 mmol/l potassium phosphate, pH = 7.4, in a final volume of 1 ml. The reaction mixture contained 0.4–0.7 mg of mitochondrial protein. The reaction was started by the addition of dye. For the measurements with PGO 0.1 mol/l borate buffer, pH = 7.6; for the measurements with TNBS 0.25 mol/l sucrose, 20 mmol/l MOPS-HCl, pH 6.6, was used.

Determination of Mitochondrial Succinate Dehydrogenase Activity

The activity measurement of mitochondrial succinate dehydrogenase was performed according to King (1967).

Protein Determination

Protein concentration was determined according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Results

Previous observations on purified L-glycerol-3-phosphate dehydrogenase have shown that SH-groups are involved in its function (Shi Shan-Ping *et al.*, 1986; Garrib and McMurray, 1986). We have therefore compared the inhibitory effect of various hydrophobic and hydrophilic sulfhydryl reagents. As demonstrated in Table I, we found similar inhibitory effect as other authors for the hydrophilic compounds used *in situ* conditions (Dawson and Thorne, 1975; Rauchová *et al.*, 1985). Most hydrophobic reagents were less effective with the exception of eosin-5-maleimide, which inhibited the enzyme up to 82%. This maleimide analog is relatively large in size, and bears several charged groups. Eosin alone, the bulky group of eosin-5-maleimide, could inhibit the enzyme activity by 52% using the same reaction conditions. All these reagent have to be used in mmol concentration range, which shows their limited specifity.

In further experiments we have tested the inhibitory effect of DABS on L-glycerol-3-phosphate dehydrogenase activity. This reagent reacts with a variety of functional groups in proteins including histidyl, tyrosyl, lysyl, and cysteinyl side chains, but DABS is a useful tool for the identification of functional groups localized on the outer surface of the inner mitochondrial membrane, because it does not penetrate biological membranes (Tinberg *et al.*, 1974; Dawson, 1974). The intactness of mitochondria was tested *via* measuring the effect of DABS on succinate dehydrogenase activity. DABS

Table I. Inhibition of L-glycerol-3-phosphate Dehydrogenase Activity by Different SH-Modifying Reagents[a]

SH reagent	Incubation time (min)	Inhibition (%)
Hydrophilic reagents		
Mersalyl	2	40 ± 7
	30	66 ± 8
p-chloromercuri-benzoate	2	38 ± 8
	30	56 ± 11
5,5′-Dithiobis(-2-nitrobenzoic acid)	2	21 ± 5
	30	32 ± 8
Hydrophobic reagents		
N-Ethylmaleimide	2	0
	30	7 ± 2
N,N′-p-Phenylenedimaleimide	2	0
	30	23 ± 5
N,N′-o-Phenylenedimaleimide	2	0
	30	26 ± 5
Eosin-5-maleimide	2	0
	30	82 ± 15
Eosin (control)	2	0
	30	52 ± 8

[a]The reaction mixture contained 10 mg/ml protein and 5 mmol/l SH modifying reagent at 20°C. The reaction was stopped after 2 or 30 min. Controls were measured in the presence of suitable solvents. All points represent the mean values of 3–4 independent experiments.

can lower its activity only in inside-out submitochondrial particles or in damaged mitochondria (Tinberg *et al.*, 1974). In our experiments this diazo compound did not affect the succinate dehydrogenase activity. Neverthless, DABS has a strong inhibitory effect on L-glycerol-3-phosphate dehydrogenase activity at low reagent concentration. It inhibits the enzyme completely and for 50% inhibition 27.6 ± 9.7 μmol DABS/l is required. Due to the low side chain specificity of DABS we have compared its inhibitory effect with that of the other, more specific reagents.

The results obtained from the chemical modification of L-glycerol-3-phosphate dehydrogenase with different specific reagents are summarized in Table II. From the data presented it is evident that only TNM (which reacts preferentially with tyrosyl amino acid side chain) and TNBS (by which lysyl residue can be modified) have inhibitory effects comparable with that of DABS. DEPC, which reacts with histidyl side chains, also inhibits the glycerol-3-phosphate dehydrogenase. PGO and DCCD modifying arginyl and carboxyl groups, respectively, had no inhibitory effects, indicating that these side chains are not involved in enzyme function.

To decide which side chains are modified by DABS we compared the inhibitory effects of TNM and TNBS with that of DABS. Even in mmol

Table II. Effect of Different Chemical Modifying Reagents on L-glycerol-3-phosphate Dehydrogenase Activity[a]

Reagent	Specificity	Concentration (mmol/l)	Incubation time (min)	Inhibition (%)
DABS	Tyr, His, -SH, $-NH_2$	0.05	2	95 ± 5
DCCD	-COOH	14.3	10	5 ± 3
PGO	Arg	2.0	10	7 ± 4
DEPC	His	0.3	10	29 ± 4
		0.5	10	36 ± 6
TNBS	Lys	0.1	30	75 ± 6
		0.3	30	83 ± 5
TNM	Tyr	0.17	2	93 ± 7

[a]Mitochondrial protein concentration was between 0.30–0.65 mg/ml. The incubation was performed in 1 ml final volume at 20°C. The incubation time means the end point of the chemical modification reactions. All points represent the mean values of 2–5 independent experiments.

concentrations of TNBS about 20% residual enzyme activity can be observed. To reach the endpoint of the inhibition by TNBS 30 min are needed (Table II). Only μmol amounts of TNM (Fig. 1) are needed to inhibit the activity of the same level as seen with DABS. The enzyme activity can be completely blocked by both reagents, and it is completed within 2 min (Table II). The concentration required for 50% inhibition by TNM is

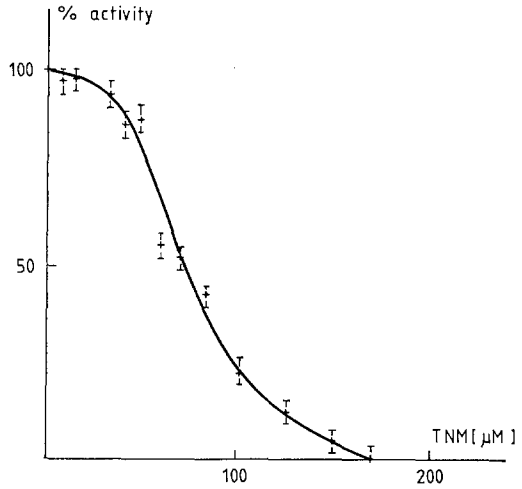

Fig. 1. The chemical modification of mitochondrial L-glycerol-3-phosphate dehydrogenase by TNM. The mitochondrial protein concentration was 0.48 mg/ml. The activity was measured when the modification was completed, 2 min after adding TNM. The modification was performed at pH = 8.0.

approximately three times as high as in the case of DABS. Titration curve of mitochondrial L-glycerol-3-phosphate dehydrogenase with TNM always has a lag phase showing that nitration starts with other components of mitochondria, but at a higher reagent concentration the enzyme inactivation occurs in a very narrow concentration range (Fig. 1).

Dawson and Thorne (1975) and Shi Shang Ping *et al.* (1986) showed that the inhibitory effect of some chemical modifying reagents is potentiated in the presence of the substrate. We found the same effect in the case of DABS and TNM inhibition. When inhibitors act in the presence of the glycerol-3-phosphate lower concentrations of the reagents are required for

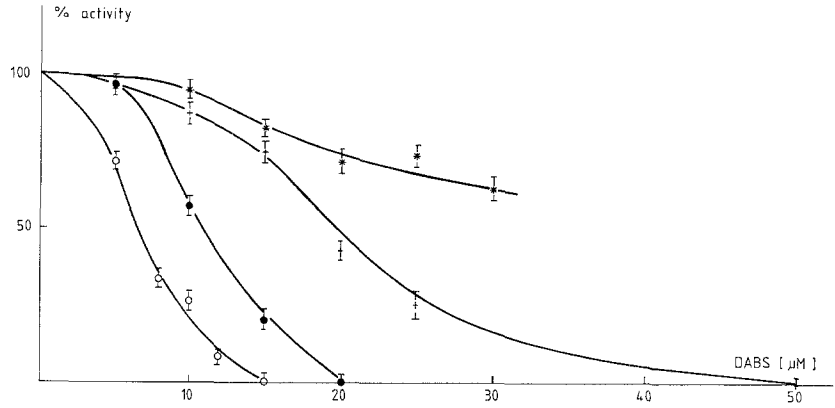

Fig. 2. The influence of different ligands on the chemical modification of mitochondrial L-glycerol-3-phosphate dehydrogenase by DABS. The mitochondrial protein concentration was 0.504 mg in 1 ml final volume. The incubation was performed in the presence of various ligands for 2 min at 20° C, and the reaction was stopped by addition of Tris-HCl, pH = 7.4 in 10 mmol/l final concentration. The enzyme activity was determined as described in Materials and Methods. Chemical modification by DABS was done: *, in calcium free medium; +, with 4 mmol/l $CaCl_2$; o, in the presence of 50 mmol/l GP; ●, in the presence of 50 mmol/l GP and 4 mmol/l $CaCl_2$.

Table III. The Influence of Different Ligands of TNM Action on L-glycerol-3-phosphate Dehydrogenase Activity[a]

Compounds	50% inhibition (μmol/l)	Number of experiments
Control	83 ± 9	4
Ca^{2+}	70 ± 2	3
sn-glycerol-3-phosphate	39 ± 3	2
Ca^{2+} + sn-glycerol-3-phosphate	52 ± 8	3

[a]Mean values ± SD are presented. The mitochondrial protein concentrations were 0.43–0.47 mg/ml. The TNM modification was done at pH = 8.0 during 2 min at 20°C in the presence of different compounds. The control means calcium free medium (1 mmol/l EDTA); the ligand concentrations were 4 mmol/l Ca^{2+} and/or 50 mmol/l sn-glycerol-3-phosphate. The activity was detected as described in Materials and Methods.

50% inhibition (Table III). The effect of glycerol-3-phosphate and/or Ca^{2+} on the chemical modification of the enzyme by DABS is shown in Fig. 2. In the presence of Ca^{2+} increased inhibition was obtained, but the effect was more pronounced in the presence of glycerol-3-phosphate. When both ligands are applied their effects are not additive. Similar results were obtained with TNM (Table III).

Discussion

Characterization of amino acid side chains essential for catalysis on enzyme molecules can help to understand the mechanism of action of ligands influencing enzyme function.

If the chemical modification is performed *in situ*, the possibility of indirect effects on the investigated proteins could arise. Because of the sensitivity of the membrane-bound enzymes for the lipid environment, the physical incorporation of different modifying reagents into the mitochondrial membrane might result in changes of enzyme activity, especially at high reagent concentrations.

Our data confirm the previous findings that the modification of SH-group(s) leads to the inhibition of L-glycerol-3-phosphate dehydrogenase activity. Our data show that the important *cys* side chain(s) are located on the cytosolic surface of the enzyme. The rather slight inhibitory effect of *cys*-modifying reagents in comparison to other specific ones might suggest that SH-group(s) are not present in the active center of mitochondrial L-glycerol-3-phosphate dehydrogenase; however, they can play a role in the enzyme function in other ways. It is in good agreement with recent findings that SH-group(s) localised on the cytoplasmic surface on the enzyme molecule participate(s) in divalent cation binding (Beleznai *et al.*, 1988).

Furthermore, mitochondria are good objects for the characterization of sideness of proteins embedded into the mitochondrial inner membrane using nonpenetrating agents as a tool. Diazonium compounds are such reagents. The strong inhibitory effect of DABS clearly demonstrates that it is possible to inhibit completely the L-glycerol-3-phosphate dehydrogenase activity by chemical modification on that part of the enzyme molecule which is exposed to the cytoplasmic phase. Since SH-groups on the surface of the enzyme molecule should be accessible as much to water-soluble mercurials as to DABS, we may conclude that in the case of the inhibitory action of DABS functional groups other than SH are modified when the enzyme activity is completely inhibited. Because of the similarity of the results obtained with TNM and DABS as modifiers, our data indicate that Tyr could be involved in L-glycerol-3-phosphate dehydrogenase function.

Ca^{2+} a modulator of L-glycerol-3-phosphate dehydrogenase and/or glycerol-3-phosphate the substrate of the enzyme increased the inhibitory actions of TNM and DABS on the enzyme activity. These effects are non-additive. The nonadditive effect of Ca^{2+} and glycerol-3-phosphate may suggest that these effectors are bound in each other's neighborhood and therefore hinder each other's binding.

The presence of Ca^{2+} induces conformational change on L-glycerol-3-phosphate dehydrogenase molecule. Due to this action the surface of the enzyme becomes more hydrophobic (Beleznai *et al.*, 1988). It is in good agreement with our results, that TNM and DABS can inhibit the enzyme activity to a higher extent in the presence of Ca^{2+}. In our experiments, the hydrophobic tyrosyl groups, which may be on in the surface of the enzyme, could become more accessible to these reagents in the presence of Ca^{2+}. In the presence of glycerol-3-phosphate the inhibitory effect of DABS and TNM is higher. It could be due to some additional effect of these reagents. They have electrophilic behavior, that is why they can preferentially modify side chain(s) on the enzyme which becomes reduced *via* glycerol-3-phosphate action. One candidate is the flavin, the prosthetic group of mitochondrial L-glycerol-3-phosphate dehydrogenase, which is in the reduced state in the presence of substrate (Cottingham and Ragan, 1980). That is why we could not exclude the possibility of the modification of FAD. On the other hand, these data suggest that the side chains modified by these reagents are not located at the glycerol-3-phosphate binding site.

The inhibitory effect of DEPC and TNBS presents further evidence that His and Lys side chains may also be involved in enzyme function. Under the reaction conditions applied, TNBS reacts with primary amino groups. It should be considered that amino head groups of the lipid components of the mitochondrial membrane components (e.g., phosphatidyl-ethanolamine) are also present. In a previous paper we have demonstrated (Amier *et al.*, 1986) that the rate of glycerol-3-phosphate oxidation is effectively regulated by altering the apparent micoviscosity of lipid membrane. TNBS might act indirectly on L-glycerol-3-phosphate dehydrogenase activity as well, by changing the properties of mitochondrial membrane.

In conclusion, in the chemical modification study of mitochondrial L-glycerol-3-phosphate dehydrogenase the electrophilic reagents such as TNM and DABS were the most effective. Ca^{2+} and glycerol-3-phosphate enhance the inhibitory action of the above reagents in a nonadditive manner.

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