

Permeability of inner mitochondrial membrane to arginine reagents

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1. INTRODUCTION

The study of permeability of the inner mitochondrial membrane to chemical agents or inhibitors has contributed much information to our knowledge of the topology and function of inner membrane proteins. In [1] we demonstrated the existence of 2 types of sulphhydryl reagents, penetrant and non-penetrant, which were subsequently utilized to localize functional thiol groups of proteins involved in mitochondrial transport and oxidative phosphorylation [2–4].

The use of α -dicarbonyl compounds which are known to react specifically with arginyl residues of proteins [5–7] has led to the clarification of the role of arginyl residues in numerous protein functions including the binding of substrate [8] or coenzyme [9], catalytic activity [10], energy conservation [11], transport [12], and the binding of protein to membranes [13].

Here we studied the permeability of the inner mitochondrial membrane (rat liver) to 4 arginine-specific reagents (methylglyoxal, butanedione, 1,2-cyclohexanedione and phenylglyoxal) to utilize these inhibitors subsequently for the study of mitochondrial membrane proteins.

(1) We measured the phase partition of each re-

agent in different solvent systems in order to compare their hydrophobic and hydrophilic properties;

- (2) Using intact mitochondria and inverted inner membrane vesicles, we tested the sensitivity of D- β -hydroxybutyrate dehydrogenase to each inhibitor. D- β -Hydroxybutyrate dehydrogenase was selected to evaluate reagent penetration because it is an amphipathic membrane protein [14] tightly bound on the matrix face of the inner membrane [1,14] and is one of the few mitochondrial enzymes consisting of a single polypeptide chain [15] known to contain an essential arginyl residue in its active site sensitive to α -dicarbonyl reagents [16,17].
- (3) To estimate the permeability of the inner mitochondrial membrane to the inhibitors, we measured the distribution of radioactive phenylglyoxal in mitochondrial and sub-mitochondrial fractions.

We found that the arginine-specific reagents could be graded from poorly penetrant, i.e. methylglyoxal, to highly penetrant, i.e., 1,2-cyclohexanedione, and that there was a correlation between their penetrability and solubility.

Abbreviations: BD, 2,3-butanedione; CHD, 1,2-cyclohexanedione; Hepes, N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; PGO, phenylglyoxal; PPO, 2,5-diphenyl-oxazone; POPOP, 1,4-di(2-methyl-5-phenyl-oxazolyl)-benzene

2. MATERIALS AND METHODS

Butanedione and 1,2-cyclohexanedione were from Merck-Schuchart, phenylglyoxal and methylglyoxal from Sigma, 2-[14 C]phenylglyoxal with spec. act. 32 mCi/mmol, stored in methanol under

nitrogen at -20°C from the Commissariat à l'Énergie Atomique, Saclay and D,L- β -hydroxybutyrate (sodium salt) and NAD^{+} grade II from Boehringer. All other chemicals were of analytical grade. All solutions were prepared in double-distilled deionized water and pH-adjusted when required.

Mitochondria which gave a respiratory control ratio between 4–5 with succinate as substrate were isolated from the livers of adult Wistar albino rats as in [18]. Submitochondrial vesicles, obtained as in [19], were 90% inverted as shown by both the cytochrome *c* affinity method [20] and the enzymatic method [21]. Protein content was determined as in [22]. Liposomes (phospholipid bilayer vesicles) were prepared as in [15] after extraction of mitochondrial phospholipids [23]. Phosphorus was estimated as in [24].

Phase partitioning of arginine reagents in water/*n*-hexane and water/1-octanol was accomplished by measurement of reagent concentration in each phase after separation in separatory funnels and pre-saturation of each phase with the other phase at room temperature. Relative concentrations of arginine reagents were measured in a double beam spectrophotometer at: 255 nm for butanedione; 264 nm for 1,2-cyclohexanedione; 212 nm for methylglyoxal; 252 nm for phenylglyoxal.

D- β -Hydroxybutyrate dehydrogenase inactivation experiments were carried out as follows:

- (1) In inverted inner membranes; submitochondrial vesicles (1.7 mg protein/ml) were preincubated for 5 min at 25°C in either 50 mM Hepes (pH 7.5) or in 10 mM Hepes, 50 mM borate (pH 7.5) when the reagent was butanedione. Inhibitor (freshly prepared in water) was added at zero time and aliquots were removed at indicated times during incubation to measure enzymatic activity.
- (2) In intact mitochondria; mitochondria (20 mg protein/ml) were preincubated for 5 min at 25°C in isotonic buffer (280 mOsm) containing either 35 mM Hepes (pH 7.5) or in 10 mM Hepes, 25 mM borate (pH 7.5) when using butanedione. Labeled or unlabeled inhibitor was added at zero time of incubation. Aliquots were removed at indicated times, quickly diluted (40-times) into a cold (0°C) isotonic medium

containing 5 mM Mes (pH 6.15) or 5 mM Mes, 25 mM borate (pH 6.15) when butanedione was the inhibitor. In all cases, sucrose was used to adjust all media to isotonicity. After centrifugation of the aliquots at $8000\times g$ for 10 min at 4°C the pellets were resuspended in the same medium and centrifuged again. After the second wash, pellets were suspended in a hypotonic medium (≈ 4 mg protein/ml) containing 5 mM Mes (pH 6.15) or 5 mM Mes, 25 mM borate (pH 6.15) when using butanedione, then sonicated for 3×30 s (at 90% maximum power of a Braun Sonic Homogenizer with a 2.5 cm diam. tip) at 0°C under nitrogen stream. Aliquots were removed for measurement of enzymatic activity and determination of protein content. The remaining sonicated suspension was ultracentrifuged at $105\,000\times g$ for 45 min in order to pellet the membranes (resuspended in 0.25 M sucrose) and to obtain soluble fractions for radioactive assays. At each step, aliquots were reserved for protein estimation yield and radioactive counting using a PPO-POPOP-toluene scintillation fluid.

D- β -Hydroxybutyrate dehydrogenase activity was measured spectrophotometrically at 334 nm ($\epsilon = 6 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) by following NADH production at 25°C . Samples (20–80 μl) containing submitochondrial vesicles ($\approx 40 \mu\text{g}$ protein) or sonicated mitochondria ($\approx 300 \mu\text{g}$ protein) were incubated for 3 min in a 1 ml cuvette in the following medium [15]: 10 mM potassium phosphate, 0.5 mM EDTA, 0.04% bovine serum albumin, 1.27% redistilled ethanol (pH 7.35) in the presence of 2 mM NAD^{+} and 2.5 μg antimycin A (final addition) to prevent NADH reoxidation by the respiratory chain. Addition of 0.1 ml of D,L- β -hydroxybutyrate to 20 mM final conc. initiated the enzymatic reaction. Borate (25 mM) was also added to assays pre-incubated with butanedione. In control assays using non-sonicated mitochondria, sucrose was added to adjust to isotonicity. Initial rates were taken in order to calculate specific activity in $\mu\text{mol NAD}^{+}$ reduced $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

Oxidative activity was measured using an oxygen polarograph equipped with a 1.8 ml cell and Clark electrode. Exact experimental details are given in table 2.

3. RESULTS AND DISCUSSION

3.1. Determination of the hydrophobic and hydrophilic properties of the arginine reagents

The phase partition of the reagents was estimated in 2 biphasic systems: a hydrophilic/hydrophobic system (water/*n*-hexane) and a hydrophilic/amphiphilic system (water/1-octanol). The data in table 1 show that all the reagents were more soluble in water than in hexane; 1,2-cyclohexanedione being the least hydrophilic. When the organic phase was 1-octanol, a solvent which contains both polar and non-polar groups and which partially mimics some of the properties of a phospholipid molecule, the solubility of the reagents increased with increasing carbon number:

methylglyoxal < butanedione < 1,2-cyclohexanedione < phenylglyoxal.

3.2. Sensitivity of D-β-hydroxybutyrate dehydrogenase to arginine reagents

The effects of the arginine reagents on D-β-hydroxybutyrate dehydrogenase activity observed after incubation of inverted inner membranes or

intact mitochondria with these compounds are presented in fig.1.

Reagent concentrations were selected to obtain strong inhibition of enzyme activity in inverted vesicles for all reagents tested. With intact mitochondria methylglyoxal (fig.1A) and phenylglyoxal (fig.1D) had little effect whereas butanedione (fig.1B) and 1,2-cyclohexanedione (fig.1C) strongly inhibited. With 1,2-cyclohexanedione, inhibition was almost the same with both types of particles.

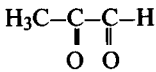
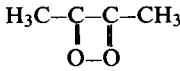
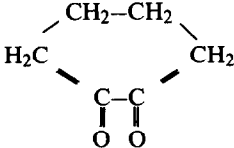
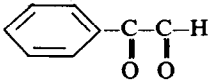
Under similar incubation conditions, D-β-hydroxybutyrate oxidase activity in intact mitochondria was very strongly inhibited (oxygen as the final electron acceptor), especially by 1,2-cyclohexanedione and phenylglyoxal (table 2). This indicates that some arginyl residues of respiratory chain components located on the outer face or inside the inner membrane had reacted with the inhibitors.

3.3. Distribution of [¹⁴C]phenylglyoxal in different mitochondrial fractions

Incorporation of phenylglyoxal into mitochondria and submitochondrial fractions was measured un-

Table 1

Phase partition of some α-dicarbonyl compounds in a hydrophilic/hydrophobic system and a hydrophilic/amphiphilic system

Compound (arginine reagents)	Name	Percentage of phase partition in	
		water/ <i>n</i> -hexane	water/1-octanol
	Methylglyoxal	88/12	79/21
	Butanedione	96/4	64/36
	1,2-Cyclohexanedione	73/27	34/66
	Phenylglyoxal	97/3	7/93

Experimental conditions: see section 2.

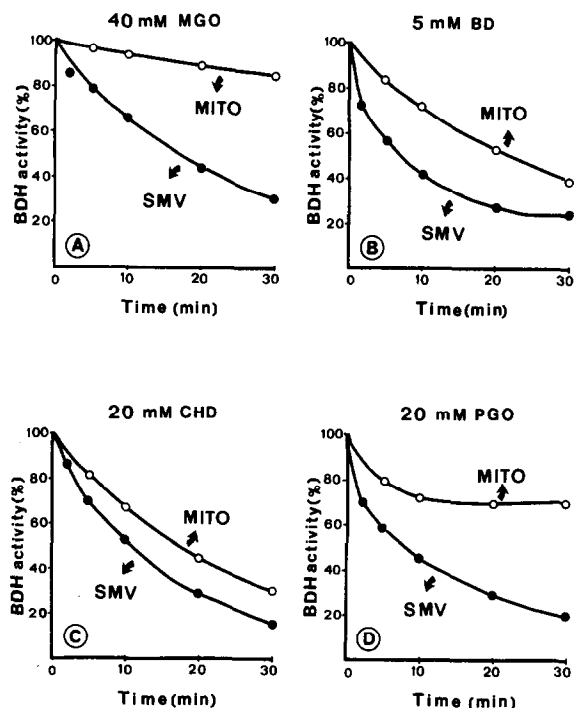


Fig.1. Inhibition of D-β-hydroxybutyrate dehydrogenase (BDH) activity by α-dicarbonyl reagents in inverted inner mitochondrial membrane and in intact mitochondria (MITO) with: (A) methylglyoxal (MGO); (B) butanedione (BD); (C) 1,2-cyclohexanedione (CHD); (D) phenylglyoxal (PGO). Experimental conditions as in section 2. D-β-hydroxybutyrate dehydrogenase activities in intact mitochondria were measured after sonication of mitochondria and were corrected as follows: (1) Taking into account the slight reversibility (~10%) of the chemical reaction observed after 2 washes of the mitochondria (results obtained with inverted inner membranes, unpublished data); (2) According to the amount of damaged mitochondria (5%). This value was obtained by measuring the ratio:

D-β-hydroxybutyrate dehydrogenase activity of
the non-sonicated mitochondria

D-β-hydroxybutyrate dehydrogenase activity of
sonicated mitochondria

Table 2

Influence of α-dicarbonyl compounds on D-β-hydroxybutyrate oxidase activity in intact mitochondria

α-Dicarbonyl compounds (mM)	Incubation (min)	D-β-hydroxybutyrate oxidase activity in state 3	
		Specific activity (n atom O · min ⁻¹ · mg protein ⁻¹)	% Inhibition ^a
Control	5	78.5	—
	30	73.5	—
BD (5 mM)	5	47.1	40
	30	19.7	74
CHD (20 mM)	5	9.8	87
	30	4.9	99
PGO (20 mM)	5	9.8	87
	30	0	100

^aIdentical results were obtained using mitochondria washed once after incubation

Experimental conditions: At the end of incubation, an aliquot containing 4 mg mitochondrial protein was added to an oxygen polarograph cell with a Clark electrode, containing an isotonic respiratory medium: 80 mM MgCl₂, 50 mM KCl, 10 mM Hepes, 25 mM borate, sucrose (pH 7.3 at 25°C). Oxygen consumption was started by addition of 10 mM D,L-β-hydroxybutyrate. State 3 was obtained by addition of 5 mM potassium phosphate and 0.15 mM ADP

Table 3

Incorporation of [^{14}C]phenylglyoxal into mitochondria and its distribution into mitochondrial fractions

Incubation (min)	[^{14}C]Phenylglyoxal incorp. (nmol/mg protein)			[^{14}C]Phenylglyoxal in mito- chondrial spaces (mM)		[^{14}C]Phenylglyoxal incorp. in liposomes ^c (nmol/ μg lipid phosphorus)
	Mito- chondria	Membranes	Soluble fraction	Extramitochon- drial space	Matrix space ^b	
0	0	0	0	20	0	0
5	19	25	17	20	5.3	—
30	45	32(1.4) ^a	26	20	8.1	2.1

^a Numbers in parentheses give nmol [^{14}C]phenylglyoxal incorporated μg lipid phosphorus due to the fact that the membrane fraction contains $\sim 23 \mu\text{g}$ lipid phosphorus/mg protein

^b Concentration of reagent within matrix space was estimated by taking into account the following: (1) the different mitochondrial spaces determined as in [25] using [^{14}C]dextran, $^3\text{H}_2\text{O}$ and [^{14}C]sucrose: $4.8 \mu\text{l}/\text{mg}$ protein for aqueous space (mitochondrial volume), $3.3 \mu\text{l}/\text{mg}$ protein for sucrose space (outer membrane plus intermembrane space), and therefore $1.5 \mu\text{l}/\text{mg}$ mitochondrial protein for matrix space [26]; (2) the relative amount of protein in soluble fraction (47%) as compared to the amount in the membrane fraction obtained after sonication of intact mitochondria

^c [^{14}C]Phenylglyoxal was incubated for 30 min with liposomes ($25 \mu\text{g}$ lipid phosphorus/ml), then the medium was extensively dialysed against 500 vol. dialysis buffer containing 5 mM Mes (pH 6.15) which was changed 3 times at 60 min intervals. See section 2 for conditions

der the conditions listed in fig.1D. The results reported in table 3 show that mitochondria, total membranes and soluble fraction all quickly incorporated [^{14}C]phenylglyoxal and that this incorporation was time-dependent. Incorporation into membranes fractions did not completely correspond to covalent binding of phenylglyoxal to arginyl residue of membrane proteins since it was shown, using liposomes preparations, that large amounts of labeled inhibitor were retained by phospholipids, even more than in mitochondrial membranes when expressed/mg lipid phosphorus.

Our measurement of intramitochondrial [^{14}C]phenylglyoxal accumulation in the matrix space indicates that the phenylglyoxal concentration in the matrix space was much lower than in the incubation medium. Under these conditions, the inhibition of D- β -hydroxybutyrate dehydrogenase activity observed in intact mitochondria (fig.1D) approximated the inhibition measured in inverted vesicles at the same concentrations of phenylglyoxal as in [16].

4. CONCLUSIONS

Butanedione and 1,2-cyclohexanedione are penetrant arginine reagents since they strongly inhibit

D- β -hydroxybutyrate dehydrogenase activity in inverted inner mitochondrial membrane vesicles and in intact mitochondria. In contrast, methylglyoxal, even at high concentration, is a relatively non-permeable reagent, in agreement with its low solubility in 1-octanol. The case of phenylglyoxal is more complex since it had little effect on D- β -hydroxybutyrate dehydrogenase activity although it was observed to accumulate in the membranes and matrix. Comparison of the [^{14}C]phenylglyoxal incorporation into membranes and liposomes indicates a strong affinity, or even a sequestration, of this reagent by the phospholipid bilayer, in agreement with the high solubility of phenylglyoxal in 1-octanol. In [27] it was concluded that butanedione and phenylglyoxal were penetrant reagents because of their inhibitory effect on the ADP-ATP translocator and the ATPase activity in beef heart mitochondria.

The arginine reagents investigated here can enter in mitochondria to different degrees, and should prove to be valuable probes in the study of topography, arrangement and sidedness of protein components or polypeptide chains of the inner mitochondrial membrane. The chemical modification of strategic arginine residues in proteins by each reagent will be different, depending on the

location of the residue either on the outer face, the inner face or inside the inner mitochondrial membrane.

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