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COMPARATIVE TITRATION OF ARGINYL RESIDUES IN PURIFIED D-β-HYDROXYBUTYRATE APODEHYDROGENASE AND IN THE RECONSTITUTED PHOSPHOLIPID-ENZYME COMPLEX

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In order to titrate and understand the role of arginyl residues of D-β-hydroxybutyrate dehydrogenase, arginyl specific reagents : butanedione, 1,2-cyclohexanedione and phenylglyoxal were incubated with three different forms of the enzyme ; native enzyme (inner mitochondrial membrane bound), purified apoenzyme (phospholipid -free) and phospholipid-enzyme complex (reconstituted active form).

After complete inactivation of the enzyme by [^{14}C]-phenylglyoxal, the number of modified arginyl residues was different : one with the lipid-free apoenzyme and three with the phospholipid-enzyme complex, suggesting a conformational change of the enzyme triggered by the presence of phospholipids.

After exhaustive chemical modification either of the apoenzyme or of the phospholipid-enzyme complex with [^{14}C]-phenylglyoxal, four arginyl residues were titrated indicating that these residues are located in the hydrophilic part of the enzyme,not interacting with phospholipids.

Reconstituted enzyme inactivated by butanedione could no longer bind a pseudosubstrate (succinate) which indicates that an arginyl residue is involved in the enzyme-substrate complex formation.

The values of second order rate constants of D-β-hydroxybutyrate dehydrogenase inactivation by butanedione and 1,2-cyclohexanedione were unchanged with the three enzyme forms, suggesting that phospholipids are not involved in the substrate binding mechanism.

INTRODUCTION

D-β-hydroxybutyrate dehydrogenase of inner mitochondrial membrane is one of the most extensively studied lipid-requiring enzyme. A few years ago, Gazzotti et al. (1) have shown that lecithins were essential for

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Abbreviations used : ApoBDH, D-β-hydroxybutyrate apodehydrogenase ; BD, butanedione, BDH, D-β-hydroxybutyrate dehydrogenase, CHD, 1,2-cyclohexanedione, MPL, mitochondrial phospholipids, PGO, phenylglyoxal.

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coenzyme binding to the purified apoenzyme. We previously showed (2) that a single cysteinyl residue is required for both enzymic function and coenzyme (NAD) binding ; reactivity of this essential cysteinyl residue towards thiol reagents was strongly decreased in the presence of phospholipids ; the active center also contains an essential histidyl residue (3).

Recently we reported (4) that rat liver D-β-hydroxybutyrate dehydrogenase (inner mitochondrial membrane bound form) was inhibited by α-dicarbonyl reagents : butanedione, 1,2-cyclohexanedione and phenylglyoxal, suggesting the involvement of at least one arginyl residue essential for catalytic activity.

In this paper we show that arginyl residues of the enzyme are more or less accessible to [^{14}C]-phenylglyoxal , depending on the form looked at (lipid-free apoenzyme or the phospholipid-enzyme complex) ; moreover, we studied whether the reactivity or accessibility of these arginyl residues was phospholipid dependent.

Binding experiments of [^{14}C] succinate (a pseudosubstrate) to the enzyme revealed that an arginyl residue is essential for substrate binding.

This work appears to be the first study which puts forward the role of arginyl residues in a phospholipid requiring enzyme.

MATERIALS AND METHODS

1-[^{14}C] succinic acid (specific radioactivity : 51 mCi/mmole) and 2-[^{14}C] phenylglyoxal (specific radioactivity : 32 mCi/mmole), stored in methanol under nitrogen at - 20°C, were obtained from the Commissariat à l'Energie Atomique, Saclay, France ; for other chemicals see (4). Radioactive and non radioactive phenylglyoxal were mixed together in methanol ; methanol was evaporated under a stream of nitrogen, and phenylglyoxal was dissolved in water. After efficient mixing, the exact concentration of phenylglyoxal solution was checked by absorbance at 247 nm (ε = 11,300 $M^{-1}.cm^{-1}$) after dilution in methanol or at 253 nm (ε = 12 600 $M^{-1}.cm^{-1}$) in water as reported by Kohlbrenner and Cross (5).

BIOLOGICAL MATERIAL

Inside-out submitochondrial vesicles from rat liver were prepared as previously described (4). Rat liver D-β-hydroxybutyrate apodehydrogenase was purified to near homogeneity (90 - 95 % pure) after phospholipase A_2 treatment of mitochondria and adsorption of released apodehydrogenase on controlled pore glass according to the method of Bock and Fleischer (6) slightly modified (7). Under optimal condition of reactivation by mitochondrial phospholipid, the enzymatic activity was usually between 30-35 µmoles NAD^+ reduced/min./mg prot. at 37°C.

Protein concentration was estimated according to Lowry et al. (8); samples containing dithiothreitol were assayed as described by Ross and Schatz (9). Total mitochondrial phospholipids and purified phospholipids were prepared from freshly isolated rat liver mitochondria as previously described (10). To reactivate the apoenzyme, the phospholipids were previously microdispersed either by sonication or by dialysis under a nitrogen stream in 20 mM Tris-HCl, 1 mM EDTA pH 8.1 (11). In experiments using α-dicarbonyl reagents, microdispersions of phospholipids were dialyzed three times against 500 volumes of 20 mM Hepes, 1 mM EDTA pH 7,5 in order to remove Tris which interferes with the reaction (12) ; phosphorus was measured according to Chen et al. (13).

CHEMICAL MODIFICATION OF D-β-HYDROXYBUTYRATE DEHYDROGENASE

The different forms of the enzyme, i.e., membrane bound enzyme in submitochondrial vesicles (1.7 mg/ml), purified apoenzyme (0,4 mg/ml) or reconstituted phospholipid-enzyme complex (0.4 mg prot./ml), were incubated at 25°C with the reagent in a buffered medium containing 50 mM Hepes, pH 7.5 or 50 mM borate, 10 mM Hepes, pH 7.5 (see legend to figures and tables). Aliquots were removed at different times and the chemical reaction was stopped by different procedures described below.

ENZYMATIC ACTIVITY MEASUREMENTS

The procedure depends on the enzyme form :

1) With the membrane bound enzyme the procedure was as previously described (4).

2) With the reconstituted phospholipid-enzyme complex, apodehydrogenase was reactivated with optimal amount of mitochondrial phospholipids (60-80 µg of lipid phosphorus/mg of apoenzyme) for 20 minutes at room temperature in a medium containing 20 mM Hepes, 1 mM EDTA, 5 mM dithiothreitol pH 7.5.

3) With the chemically modified apodehydrogenase, the aliquot was diluted ten times in a medium containing mitochondrial phospholipids (60-80 µg of lipid phosphorus/mg of apoenzyme), 20 mM Mes, 1 mM EDTA, 5 mM dithiothreitol pH 6.15 and incubated for 20 minutes at room temperature.

Enzymatic reaction was started by the addition of an aliquot containing either 25 µg protein of submitochondrial vesicles in 15 µl or 4 µg protein of enzyme-phospholipid complex in 100 µl to a cuvette containing the assay mixture preequilibrated at 25°C according to Bock and Fleischer (6).

[^{14}C]-PHENYLGLYOXAL INCORPORATION MEASUREMENT

[^{14}C]-phenylglyoxal (1 to 5 mM) was added to apoenzyme or phospholipid-enzyme complex previously equilibrated at 25°C for two minutes in the above indicated medium ; the incubation varied from 2 to 60 minutes. Controls were done at the same time : 1) without [^{14}C]-phenylglyoxal, 2) with the same amount of [^{14}C]-phenylglyoxal as in the assay but without protein ; for each time, an aliquot was removed and used for enzymatic activity measurement ; another aliquot was transferred at 0°C into a medium containing the same volume of 50 mM Mes, pH 6.15. This aliquot was extensively dialyzed in treated dialysis tubing (14) for 3 hours at 4°C against 500 volumes of 2,5 mM Mes, 50 mM Borate, 1 mM dithiothreitol pH 6.15 changed 3 times. After dialysis the samples were used to measure enzymatic activity, protein concentration and [^{14}C] -phenylglyoxal incorporation. With apoenzyme, the incorporation was measured directly using PPO-POPOP-

toluene scintillation coktail ; with the phospholipid-enzyme complex, it was necessary to eliminate the non-covalently bound [^{14}C]-phenylglyoxal retained in the phospholipid bilayer : after 10 minutes of acidic precipitation in $HClO_4$ 7 % (V/V) at 0°C the sample was centrifuged for 5 minutes at 0°C in a clinical centrifuge, then 300 µl of absolute ethanol at 0°C (for about 100 µg protein per assay) were added to the pellet ; after vortexing, the sample was centrifuged again and extraction with ethanol was repeated once more. Finally the pellet was dissolved overnight at room temperature in 400 µl of solubilizing mixture containing 3.75 % cholate, 0.25 % sodium dodecyl sulfate, 0.3 M Na_2CO_3 and 7.5 mM NaOH. Aliquots were used for radioactivity counting, and protein assay. By substraction of the blanks, and knowing the specific activity of the aqueous [^{14}C]-phenylglyoxal solution and the amount of protein per assay, the incorporation of labeled phenylglyoxal into D-β-hydroxybutyrate dehydrogenase was expressed per single chain of Mr 31 000 as previously evaluated (7).

AMINO ACID ANALYSIS

Apoenzyme (10 nmoles) was hydrolyzed for 24 h in 6 M HCl at 110°C in the presence of 1 % thioglycolic acid. The number of arginines was estimated using a Beckman 120 C amino acid analyzer, and the number of arginines modified was deduced from the decrease of the ratio of arginine to phenylalanine.

REMOVAL OF FREE [^{14}C]-SUCCINATE FROM ENZYME BOUND [^{14}C]-SUCCINATE

We followed the centrifugation-filtration method described by Penefsky (15). Samples (140 µg of phospholipid-enzyme complex in 350 µl) were put on the top of a sepharose 6 B column, in a 1 ml disposable plastic seringue, previously equilibrated at 25°C in a buffer containing 50 mM borate, 10 mM Hepes, pH 7.5 ; the column was centrifuged for 2 minutes at 900 g in a clinical centrifuge and the filtrate obtained was used for radioactivity counting, protein and lipid phosphorus estimations.

RESULTS AND DISCUSSION

According to our previous results suggesting the presence of at least one arginyl residue in the active site (4), we used a labeled arginyl reagent, [^{14}C]-phenylglyoxal, in order to titrate the number of modified residues under different conditions. Figure 1 shows [^{14}C]-phenylglyoxal incorporation in the enzyme during the inactivation process. By extrapolation, a stoichiometry of about 2 and 6 moles of [^{14}C]-phenylglyoxal per single chain Mr 31 000 was found with lipid-free apoenzyme and phospholipid-enzyme complex respectively. These results suggest that phospholipids unmask two more arginyl residues or increase their reactivity as a consequence of a conformational change of the enzyme triggered by phospholipids. This is in agreement with Mc Intyre et al. (16) who found using a circular dichroism technique that phospholipids induced a small conformational change of the beef heart enzyme.

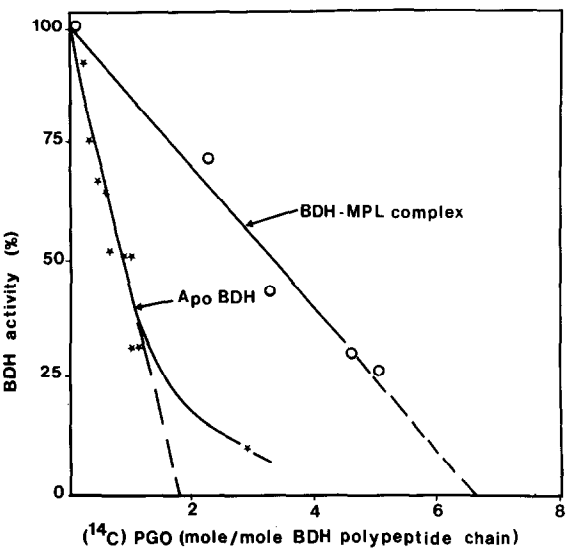

Figure 1 : Stoichiometry of inactivation of D-β-hydroxybutyrate dehydrogenase by [^{14}C] phenylglyoxal incubated with apoenzyme or with phospholipid-enzyme complex

Enzyme or phospholipid-enzyme complex was preincubated at 25°C in a medium containing 50 mM Hepes, 5 mM dithiothreitol pH 7.5. Chemical modification was initiated by addition of 2 mM [^{14}C] phenylglyoxal with apodehydrogenase or 5 mM [^{14}C] phenylglyoxal with phospholipid-enzyme complex. At different times (between 2 to 60 minutes) aliquots were removed and treated as described in experimental procedures for enzymatic activity measurement, protein assay and [^{14}C] incorporation. The remaining D-β-hydroxybutyrate dehydrogenase activity was plotted against [^{14}C] phenylglyoxal incorporated.

ApoBDH : D-β-hydroxybutyrate apodehydrogenase
BDH-MPL complex : Reconstituted phospholipid-enzyme complex

On the basis of Takahashi's work (12), it has generally been assumed that phenylglyoxal reacts with arginyl residues to give an adduct containing two phenylglyoxal per arginine. If this was the case with D-β-hydroxybutyrate dehydrogenase, the data in Figure 1 would indicate that only one arginyl residue in the apoenzyme was modified. However, there are a number of instances in which a 1 : 1 ratio of phenylglyoxal to arginyl residues has been found (17, 18). Therefore, the effect of inactivation by phenylglyoxal on the arginine content of the enzyme was measured. The data of Table I show that one arginine is lost on inactivation of the apoenzyme. Table II gives the number of arginyl residues titrated under different experimental conditions. When titration concerns the inactivation phase only, we found one arginyl residue titrated with the apodehydrogenase, while three residues were found with the phospholipid-enzyme comple

Table I

Number of free arginine residues after treatment of D-β-hydroxybutyrate apodehydrogenase by phenylglyoxal

Enzyme	Percentage of inhibition	Number of arginine residue per mole of enzyme	Variation in arginyl residue
Control	0	12.7	-
Phenylglyoxal modified apoenzyme (3 min.)	60	12.1	- 0.6
Phenylglyoxal modified apoenzyme (10 min.)	85	11.9	- 0.8

The apoenzyme (0.4 mg/ml) was modified with 3 mM phenylglyoxal in 50 mM Hepes, 5 mM dithiothreitol, pH 7.5 at 25°C. One aliquot (10 µl) was assayed for enzymatic activity and another one (1 ml) was dialyzed for 3 hours against 500 volumes of 2.5 mM Mes, 1 % thioglycolic acid, pH 6.15 at 4°C changed 3 times. After dialysis, aliquots were removed for enzymatic activity and for amino-acid analysis.

With a very large excess of [^{14}C]phenylglyoxal (1100 molar excess) and a long period of incubation, four arginyl residues were titrated either with apodehydrogenase or with the phospholipid-enzyme complex.

Table II

Number of arginyl residues titrated by [^{14}C]phenylglyoxal with different forms of D-β-hydroxybutyrate dehydrogenase under various experimental conditions

Experimental conditions	Molar ratio (mole of arginyl residue/mole of enzyme)[a]
Inactivation stoichiometry	
Apoenzyme (non active form)	1
Phospholipid-enzyme complex	3
Maximum titration with excess [^{14}C] phenylglyoxal	
Apoenzyme	4
Phospholipid-enzyme complex	4

The numbers of arginyl residues titrated during the inactivation phase were obtained from experiments reported in Figure 1.

Maximum modification was obtained by incubation of 15 mM [^{14}C] phenylglyoxal for 4 hours with apoenzyme or phospholipid-enzyme complex (0.4 mg prot./ml) in 50 mM Hepes, 3 mM dithiothreitol pH 7.5 at 25°C.

After these treatments, enzymatic activity was completely lost in all experiments. Samples were treated as indicated in experimental procedures for protein assay and radioactivity couting.

(a) Values estimated using a stoichiometry of 2 phenylglyoxal/arginyl residue according to the amino acid analysis experiment (cf Table I)

Table III

Effect of butanedione on [^{14}C] succinate binding in D-β-hydroxybutyrate dehydrogenase

Experimental conditions	% of residual activity	2 mM NAD^+ + 10 mM [^{14}C] succinate (b)	[^{14}C] succinate corrected radioactivity	% of binding
Control	100	+	420	100
5 mM butanedione (40 minutes)	11	+	40	10

The reconstituted phospholipid-enzyme complex (140 µg of enzyme) was preincubated for 2 minutes at 25°C in the following buffer : 10 mM Hepes, 50 mM borate, 3 mM dithiothreitol pH 7.5. Chemical modification was initiated by addition of 5 mM butanedione. After 40 minutes of reaction, an aliquot was removed for enzymatic activity measurement as described in experimental procedures, then 2 mM NAD^+ and 10 mM [^{14}C] succinate (575 dpm/nmole) were added to the assay mixture. After two minutes, separation and estimation of free and bound [^{14}C] succinate were performed according to Penefsky's method (see experimental procedures).

Under the same conditions three controls were done : [^{14}C] succinate alone ; [^{14}C] succinate + MPL (liposomes without enzyme) and unmodified BDH-MPL complex + [^{14}C] succinate.

This indicates that these arginyl residues are located in the hydrophilic part rather than in the hydrophobic one of the enzyme.

Results not reported here have shown a strong decrease of [^{14}C] phenylglyoxal incorporation when butanedione or 1,2-cyclohexanedione were preincubated before the addition of [^{14}C] phenylglyoxal, indicating that these three reagents compete for the same residue.

According to our previous results (4) showing that a pseudo-substrate (methylmalonate) protected the enzyme against inactivation by arginyl specific reagents, we studied the effect of methylmalonate on [^{14}C]-phenylglyoxal incorporation into the phospholipid-enzyme complex ; we found that methylmalonate decreased phenylglyoxal incorporation (not shown) ; however the decrease is lower than expected due to the fact that methylmalonate partially protects against inactivation (see ref. 4) and that the phospholipid-enzyme complex reacts with 4 more phenylglyoxal, whilst the incorporation of only two phenylglyoxal is involved in the inactivation process (see fig. 1 and the text).

According to these results, and taking into account the fact that methylmalonate strongly protects against butanedione inactivation (4)

Table IV

Comparison of inactivation rate constants of D-β-hydroxybutyrate dehydrogenase (in different forms) with α-dicarbonyl compounds

Different enzyme forms	k_2 (second order rate constant) at 25°C pH 7.5	
	BD	CHD
	$(mn^{-1}.M^{-1})$	$(mn^{-1}.M^{-1})$
Native enzyme (membrane bound)	9	0.8
Phospholipid-enzyme complex (reconstituted enzyme)	9	1
Apoenzyme (non active form)	8	1

The rates of inactivation (expressed as the second-order rate constant) were obtained by incubation of enzyme, in different forms, with α-dicarbonyl compounds at different condentrations in the following incubation medium : 50 mM Hepes, 3 mM dithiothreitol pH 7.5 or 50 mM Borate, 10 mM Hepes, 3 mM dithiothreitol pH 7.5 with butanedione at 25°C. At different times, aliquots were removed and treated for enzymatic activity measurements as described in experimental procedures. For each inhibitor concentration the percentage of remaining activiy was plotted against time (semi-log plot) in order to obtain the pseudofirst-order rate constant values (see ref. 4).

we studied the effect of another pseudosubstrate (19), succinate, available in radioactive form, on enzyme inactivation by butanedione.

Table III shows that when the enzyme is 89 % inactivated with butanedione, the [^{14}C]-succinate binding is decreased by 90 %.

These results indicate that the essential arginyl residue is directly involved in the substrate-enzyme complex formation.

In order to check whether phospholipids modify the chemical reactivity of the essential arginyl residue, we measured the second order rate constants of inactivation with the different forms of the enzyme ; as shown in Table V, the values of these constants are roughly the same whatever the enzyme form used.

This indicates that phospholipids do not affect the reactivity of the essential arginyl residue and consequently do not interfere with the substrate binding in contrast to the direct role of phospholipids in the coenzyme binding, as shown by Gazzotti et al. (1).

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