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SUCCINATE DEHYDROGENASE

II. THE EFFECT OF PHOSPHOLIPASES ON PARTICULATE AND SOLUBLE SUCCINATE DEHYDROGENASE

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SUMMARY

1. The activity of beef-heart succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) in particulate preparations is not affected by phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) but is inhibited by the action of phospholipase C (phosphatidylcholine cholinephosphohydrolase EC 3.1.4.3), and of phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4).

2. Washing of the preparation treated with phospholipase does not relieve the inhibition. Succinate dehydrogenase solubilized and purified from particulate preparations digested with phospholipase C shows a much lower activity than controls purified from undigested starting material.

3. Phospholipid hydrolysis in the particle is nearly complete after treatment with phospholipase A and C and only partially complete after treatment with phospholipase D.

4. Side reactions of the phospholipase C preparation have no detectable influence under our experimental conditions.

5. Soluble succinate dehydrogenase is not affected by the action of phospholipases, even if phosphatides are added to the mixture before treatment with phospholipases.

6. Except for phosphorylcholine, the products of phospholipase-catalyzed reactions do not affect the activity of soluble succinate dehydrogenase. Phosphorylcholine is a moderate mixed-type inhibitor and is only partly responsible for the inhibition following treatment with phospholipase C. It is suggested that phospholipases alterate the particulate flavoprotein by disrupting phospholipid organization in the membrane.

7. The inhibition by 2-thenoyltrifluoroacetone of succinate dehydrogenase activity is strongly decreased after treatment of the particles with any phospholipase.

INTRODUCTION

In previous work we studied the effect of various phospholipids on soluble beef-heart succinate dehydrogenase[1]. We found that addition of phosphatides gives the soluble enzyme properties which it had in particulate form.

We also investigated the interaction between phosphatides and intact succinate dehydrogenase in the respiratory particle. A first approach was to extract phosphatides from particulate preparations[2]. In this paper we are reporting experiments in which phosphatides were split by various phospholipases and the modifications in succinate dehydrogenase activity of digested particles were recorded. The effect of phospholipases on soluble succinate dehydrogenase was studied as well. A preliminary report has appeared[3].

MATERIALS AND METHODS

Phospholipase A was from Naja Naja venom (Koch-Light). Phospholipase C from *Clostridium welchii* was Sigma Type I. Phospholipase D from cabbage was Sigma Type II. Phosphorylcholine (calcium salt), dioleyl glycerol, dipalmitoylglycerol and tripalmitoylglycerol were from Sigma. Sialic acid was from Nutr. Bioch. Corp. The glycoprotein isolated from sheep submaxillary gland was kindly given by Dr. TETTAMANTI[4].

Glycerides[5] and phosphatides[6,7] were analyzed by thin-layer chromatography. Ninhydrin and Dragendorff[8] sprays were used to characterize, respectively, amino- and choline-containing compounds. Sialic acid was determined according to CASSIDY *et al.*[9]. All other materials and methods are those mentioned in a previous paper[1].

Mitochondria were prepared from beef heart and fragmented as described earlier[1]. Keilin–Hartree heart-muscle preparations were made by the procedure of SLATER[10] as modified by DERVARTANIAN[11]. Succinate dehydrogenase was purified by the DERVARTANIAN AND VEEGER[12] in the presence of 40 mM succinate.

Succinate dehydrogenase activity was assayed spectrophotometrically using phenazine methosulfate and 2,6-dichlorophenolindophenol as sequential acceptors[1].

Phospholipases were acted using the following systems: phospholipase A according to MAGEE AND THOMPSON[13]; phospholipase C according to VAN DEENEN *et al.*[14] and phospholipase D according to DAVIDSON AND LONG[15]. Succinate (40 mM) was added to the media when soluble succinate dehydrogenase was treated with phospholipase.

Following the incubation period, the phospholipase reaction was stopped by the addition of EDTA and/or the reaction mixture was diluted in 60 mM phosphate buffer (pH 7.6) for assaying dehydrogenase activity or for activation. In some experiments the phospholipases were not stopped; then the assays following treatment with phospholipase C were carried out in 60 mM imidazole buffer (pH 7.6). Particulate succinate dehydrogenase preparations were activated prior to assay by incubating them for 20 min at 25° in 40 mM succinate and 60 mM potassium phosphate buffer (pH 7.6). The soluble dehydrogenase needs no activation since, when prepared and kept in the presence of succinate, it has already maximal activity[16].

The hydrolysis of phospholipid in fragmented mitochondria was studied in the

same conditions as for digestion of particles. It was followed in an automatic recording titrimeter by titrating with 0.1 M NaOH at the pH of the buffer the acid liberated. Titration of phosphorylcholine gave $pK' = 5.5$ for the second dissociation of the phosphate group. The compound is therefore fully titrated in the above conditions. Titration after phospholipase D digestion is described in ref. 17. The reaction was terminated by extracting with chloroform and after acidifying with diethyl ether. The extracts and the aqueous residue were analyzed by thin-layer chromatography. Phosphorylcholine liberated by phospholipase C and residual phospholipids were estimated by determining organic phosphate, respectively, in the water phase and in the chloroform extract.

The neuraminidase activity of the phospholipase C preparation was assayed using glycoprotein as substrate[9]. The effect of neuraminidase present in the preparation on fragmented mitochondria was assayed under the conditions for treatment with phospholipase C.

Proteolytic activity in the phospholipase C preparation was assayed by treating in a volume of 5 ml 18 mg horse hemoglobin with 3 mg phospholipase in the conditions described for digestion of particles. Ninhydrin-positive compounds were assayed in the supernatant from trichloroacetic acid precipitation according to ROSEN[18].

RESULTS

Phospholipid hydrolysis in particles during phospholipase action

Table I shows the quantitative effects of different types of phospholipases on the phospholipids of fragmented mitochondria. Assuming that one ester bond is available to phospholipase A for each atom of phospholipid phosphorus in the preparation, the hydrolysis is nearly complete. In thin-layer chromatograms, all phospholipids present in control samples disappear after digestion, except a spot with an R_F similar to cardiolipin and spots running near the front in the alkaline solvent of DAVISON AND GRAHAM-WOLFAARD[6].

TABLE I

THE ACTION OF PHOSPHOLIPASES ON PHOSPHOLIPIDS OF FRAGMENTED MITOCHONDRIA

Fragmented beef-heart mitochondria were incubated in the conditions given in the text with phospholipases, or the controls without phospholipases added. In all cases the reaction was completed within 10 min. The extent of phosphatide hydrolysis was measured by determining titrimetrically the acid produced and, for phospholipase C water-soluble organic phosphate liberated. These values are referred as percent of the total phospholipid phosphorus in fragmented mitochondria (14.2 μg phosphorus per mg protein was the average in our preparations).

Phospholipase	*Phospholipase added (mg/mg protein of fragmented mitochondria)*	*Fragmented mitochondria digested (mg protein)*	*Incubation time (min)*	*Phospholipid hydrolysis (%)*	
				From acid liberated	*By other methods*
Phospholipase A	0.25	20	30	97.7	
Phospholipase C	0.17	30	15	96.4	90.1
Phospholipase D	0.20	100	15	27.4	

With phospholipase C the hydrolysis is nearly complete. Amounts of water-soluble organic phosphate formed agree with data from acid liberated. In thin-layer chromatograms, phospholipids present in control samples disappear, except a spot with an R_F similar to cardiolipin, a ninhydrin-positive spot with an R_F similar to phosphatidylserine and a spot running near the front. The phospholipase C preparation was found to contain some neuraminidase activity. However upon treatment of fragmented mitochondria with phospholipase C, no free sialic acid was formed. Under the conditions used for the assay of proteolytic activity, no ninhydrin-reactive substances were produced.

Effect of phospholipase action on particulate succinate dehydrogenase

The results shown in Table II remain essentially the same when incubation times or the ratio between phospholipases and succinate dehydrogenase are varied,

TABLE II

EFFECT OF PHOSPHOLIPASES ON THE ACTIVITY OF PARTICULATE SUCCINATE DEHYDROGENASE

Fragmented heart mitochondria were preincubated in a N_2 atmosphere under the conditions given below with phospholipases, or the controls without phospholipase added. They were then activated for 20 min at 25° in 40 mM succinate and 60 mM potassium phosphate buffer (pH 7.6). Succinate dehydrogenase activity was then assayed as described in the text. It is reported as mmoles succinate oxidized per min per g protein at 25° and infinite phenazine concentration (v_{max}).

Phospholipase	*Phospholipase added (mg/mg succinate dehydrogenase prep.)*	*Conditions at preincubation*			*Succinate dehydrogenase activity*		
		Buffer	*Concn. (mM)*	*Time (min)*	*Control**	*Treated with phospholipase*	
					v_{max}	v_{max}	*% of control*
Phospholipase A	0.18	Phosphate	60	90	0.620	0.775	125
Phospholipase C	0.17	Tris	60	30	0.452	0.138	31
Phospholipase D	0.20	Acetate	100	30	0.560	0.388	69

* The activity of controls is in the average 15% less than in fragmented mitochondria assayed without preincubation.

when succinate is present during treatment with phospholipases or when phospholipases act during activation or assay of the dehydrogenase. The small increase in activity after treatment with phospholipase A is usually observed.

The following experiments were done to establish if the inhibition of particulate succinate dehydrogenase was caused by the presence of products of phospholipase action which might be removed by washing or by purification of the dehydrogenase, or was due to a permanent alteration of the flavoprotein.

(a) Fragmented mitochondria digested with phospholipase C were sedimented and then washed with 60 mM phosphate buffer (pH 7.6). Succinate dehydrogenase activity assayed before washing was 28% and after washing 33% of that of undigested controls. Controls were not modified by washing.

(b) Particulate preparations were digested with phospholipase C and then

succinate dehydrogenase was solubilized and purified. Dehydrogenase activity was assayed at various steps of the complete purification scheme and was found to be always 60–70% less than in controls treated in the same manner without the addition of phospholipase C.

The inhibition by 2-thenoyltrifluoroacetone of succinate dehydrogenase activity is affected to a different extent by the various types of phospholipases (Table III).

TABLE III

EFFECT OF PHOSPHOLIPASE ACTION ON THE INHIBITION BY 2-THENOYLTRIFLUOROACETONE OF PARTICULATE SUCCINATE DEHYDROGENASE

Fragmented mitochondria were preincubated with phospholipases in the conditions shown. They were then suspended in 60 mM phosphate buffer (pH 7.6) containing 40 mM succinate and succinate dehydrogenase was activated by incubating anaerobically 20 min at 25°. Where indicated 2-thenoyltrifluoroacetone (0.2 mM) was then added and the preparation allowed to stand 5 min at 25°. Succinate dehydrogenase was then assayed as usual. The activity is reported as mmoles succinate oxidized per min per g protein at 25° and infinite phenazine concentration (v_{max}). 2-Thenoyltrifluoroacetone in the assay was 0.1 mM.

Additions	*Phospholipase added (mg/mg succinate dehydrogenase prep.)*	*Conditions at preincubation*			*Succinate dehydrogenase activity*		
		Buffer	*Concn. (mM)*	*Time (min)*	*Control, no 2-thenoyltrifluoroacetone added* v_{max}	*2-Thenoyltrifluoroacetone added* v_{max}	*% of control*
None	0	Phosphate	60	0	0.624	0.408	65
Phospholipase A + 10 mM EDTA	0.11	Phosphate	60	15	0.534	0.352	66
Phospholipase D + 100 mM EDTA	0.11	Acetate	100	15	0.518	0.325	63
Phospholipase A	0.15	Phosphate	60	60	0.632	0.556	88
Phospholipase C	0.17	Tris	60	30	0.158	0.155	98
Phospholipase D	0.20	Acetate	91	30	0.420	0.350	83

Effect of phospholipase action on soluble succinate dehydrogenase

The activity of soluble succinate dehydrogenase is not affected by the action of any phospholipase. Proper controls were run to account for thermal inactivation.

We considered the possibility that phospholipase C has no effect on the soluble dehydrogenase because the amount of endogenous phospholipids in purified dehydrogenase preparations is very small. To this purpose, we incubated soluble succinate dehydrogenase, in the presence of mitochondrial phospholipids, with phospholipase C or else reacted phospholipids with phospholipase C and then added this mixture to the dehydrogenase at the moment of assaying the activity. About 10% inhibition of succinate dehydrogenase activity was observed only where very high lipid concentrations were used (5.8 mg phospholipid per mg protein). Under these conditions, a solid phase containing diglycerides separates.

Effect on soluble succinate dehydrogenase of the products of phospholipase-catalyzed reactions

Lysolecithin or phosphatidic acid, 0.1 mg phosphorus per mg protein, or 5 mM choline incubated with the soluble dehydrogenase 30 min at 0 or 37° or added at the

moment of the assay have no sizeable effect on the activity of the enzyme. Phosphorylcholine either calcium or sodium salt, is a weak mixed-type inhibitor of soluble succinate dehydrogenase. Added in inhibitory concentrations it is completely removed after one precipitation of the enzyme with $(NH_4)_2SO_4$ and the inhibition disappears. Diolein, dipalmitin, triolein and tripalmitin were tested in a variety of conditions. The glycerides were dispersed in 60 mM phosphate buffer (pH 7.6) by sonicating, and amounts varying from 0.18 to 1.4 mg glyceride per mg dehydrogenase protein were either preincubated 15 min at 0 or 37° with the flavoprotein or were added at the moment of assay. No effect was found on the activity of succinate dehydrogenase.

Binding of the dehydrogenase to the membrane and phospholipase action

Preparations digested with phospholipase C or D were centrifuged 30 min at 144 000 × *g*. The total and specific dehydrogenase activity in the supernatant were, respectively, 4 and 2% of those in the pellet, with only a minor increase as compared to controls. Phospholipase A does not introduce any modification. In the conditions of these experiments soluble succinate dehydrogenase is not sedimented.

DISCUSSION

In previous studies we have shown that solvent extraction modifies the properties of particulate succinate dehydrogenase[1,2]. The enzyme in acetone powders exhibits catalytic center activity and stability similar to the soluble flavoprotein. Solvents however labilize to a high extent the binding of the flavoprotein to the inner mitochondrial membrane and mild extraction solubilizes the dehydrogenase[2]. It is known that the catalytic center activity in succinate oxidation decreases when the flavoprotein is disconnected from the respiratory system by treatment with butanol or with cyanide[1,19]. In our case, modifications observed cannot be attributed to solubilization of the dehydrogenase. In this respect, the use of phospholipases seems to represent a closer approach than solvent extraction to the flavoprotein integrated within the respiratory assembly.

Side reactions of the phospholipase preparation seem not to play a role in our experimental conditions, and the effects observed after treatment with phospholipase C result from phospholipid breakdown. EDTA which binds Ca^{2+}, necessary for phospholipase C and D activities, abolishes all the effects of the phospholipase preparations on particulate succinate dehydrogenase. Phospholipases have no effect on the soluble dehydrogenase, which has already undergone a lipid extraction.

It seems unlikely that the loss of catalytic center activity is due to the presence of inhibitors derived from the phospholipase treatment. This, even if one assumes that during phospholipase action, substances might be produced in locally high concentrations, which irreversibly modify the dehydrogenase, whereas, under our experimental conditions, they have no effect on the catalytic activity of the soluble enzyme.

We prefer to suggest that within the inner mitochondrial membrane, phospholipids and other hydrophobic materials are important in determining the micro-environment of the flavoprotein and in stabilizing its native active conformation. These conditions may be severely affected by phosphatide cleavage resulting in alterations in the flavoprotein. This hypothesis accounts also for the effects observed after solvent extraction[1,2]. The reactivation obtained after addition of phospholipid to solvent

extracted succinate dehydrogenase or to the solubilized dehydrogenase from particles treated with phospholipase C (ref. 17) shows that the alterations in question are at least in part reversible. Phospholipase A and C have been used in other cases to modify the interactions of phosphatides with membrane-bound enzymes[20,21]. The effects appear not to be due to the products of phospholipase action but to a disturbance in the normal interrelations of protein and lipid in the membrane[20,21].

In previous work we suggested that phospholipids facilitate the interaction of 2-thenoyltrifluoroacetone with nonheme iron in the flavoprotein[1]. The present results showing that phospholipase action decreases or abolishes 2-thenoyltrifluoroacetone inhibition, confirm these previous views. Phospholipase A affects the inhibition of the particle-bound enzyme by 2-thenoyltrifluoroacetone although it does not influence the catalytic activity of the dehydrogenase. Also in soluble succinate dehydrogenase, phospholipids differently affect 2-thenoyltrifluoroacetone inhibition and catalytic activity[1]. These data suggest that different phosphatides may separately influence several different functions of succinate dehydrogenase.

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