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DIHYDRO-OROTATE DEHYDROGENASE OF *ESCHERICHIA COLI* K12: EFFECTS OF TRITON X-100 AND PHOSPHOLIPIDS

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SUMMARY

When dihydro-orotate dehydrogenase, a membrane-bound enzyme of *Escherichia coli* K12, is treated with the nonionic detergents Triton X-100 or Brij 35 it loses oxidase activity, but its activity (V) with the artificial electron acceptor dichlorophenolindophenol (DCIP) is increased when measured in the presence of the detergent. Triton X-100 does not modify the $K_{m,\,app}$ of the enzyme but it changes the inhibition by the product orotic acid from a mixed to a competitive type. Triton X-100 also solubilizes the enzyme to give a particle of markedly smaller size and lower density. The solubilized enzyme is stimulated by phosphatidylethanolamine and diphosphatidylglycerol but not as much as by Triton X-100. Phospholipase A_2 treatment results in loss of activity. The latter effect seems to be due partly to inhibition by fatty acids and partly to the loss of phospholipids. Triton X-100 restores activity.

INTRODUCTION

Dihydro-orotate dehydrogenase (EC 1.3.3.1) of *Escherichia coli* is a membrane-bound enzyme that catalyzes the synthesis of orotate from dihydro-orotate in the pyrimidine biosynthetic pathway. Taylor and Taylor[1] have shown that this enzyme is associated with the oxidative chain of the cell. Thus, it forms a link between biosynthetic and respiratory systems. The work of Yates and Pardee[2] indicated that the cellular level of this enzyme is controlled by the pyrimidine nucleotide endproducts. The regulation of the activity of the enzyme has not received much attention. The study reported here was undertaken to elucidate the relationship between dihydro-orotate dehydrogenase activity and the enzyme's association with the cytoplasmic membrane.

The particulate enzyme was subjected to various treatments that might be expected to modify its activity as an oxidase and/or as a primary dehydrogenase with the artificial electron acceptor dichlorophenolindophenol (DCIP). Treatment with the

Abbreviation: DCIP, dichlorophenolindophenol.

nonionic detergent Triton X-100 which has has already been reported to stimulate this[3] and other (*e.g.* refs 4–7) particulate enzymes turned out to be of particular interest because (1) dehydrogenase activity in its presence appeared to give a better indication of the amount of enzyme present than activity in its absence, and (2) the detergent solubilizes the enzyme and allows further purification.

This paper describes the effects of Triton X-100 and of some lipids on dihydro-orotate dehydrogenase of *E. coli* K12.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The *E. coli* (pyr E^-) used in this work was a transduction derivative of strain AT1243 (*met⁻ pyr⁻*)kindly provided by J. Puig. It was grown at 37 °C in 100 l batches in an Amsco Biogen with forced aeration on medium containing 50 mM potassium phosphate, pH 7.0, 0.2% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2$, 0.2% Difco Casamino acids (technical), 0.2% glucose (autoclaved and added separately), and 800 mg uracil (a growth-limiting concentration). The cells were harvested by centrifugation 2 h after growth had stopped in order to allow derepression of the enzyme[8].

Enzyme preparation

The cell paste weighing about 150 g (wet wt) was suspended in 500 ml of 0.04 M Tris–HCl buffer (pH 7.6) containing 1 μg each of deoxyribonuclease (Worthington) and ribonuclease (Koch–Light), and broken in a French press (American Instrument Co., Inc.) at 6 tons per square inch. When the suspension was no longer viscous, neutral EDTA was added to 1 mM. Cell debris and heavy particles were removed by successive centrifugations at 20 000 $\times$ *g* for 30 min (Servall) and at 40 000 $\times$ *g* (Spinco rotor Ti50) for 45 min. Enzyme of the highest specific activity was found in the pink upper layer of the pellet obtained by centrifugation at 100 000 $\times$ *g* for 1 h. This was resuspended and washed repeatedly in Tris–EDTA buffer until a pellet was obtained that had no lower layer of clear colorless material. Lower and higher speed centrifugations sedimented enzyme of lower specific activity that could be purified further if necessary. The particle preparation thus obtained has both dihydro-orotate dehydrogenase and dihydro-orotate oxidase activity. The enzyme was solubilized by addition of Triton X-100 (British Drug Houses and Koch–Light) to give 1 mg detergent to 3 mg of protein/ml. The supernatant obtained by centrifugation at 165 000 $\times$ *g* for 2 h was fractionated with $(NH_4)_2SO_4$. The protein precipitating between 30 and 50% saturation was resuspended in Tris–HCl–EDTA–0.1% Triton X-100 and filtered at room temperature through an agarose column (A-5, 200–400 mesh of Biorad Laboratories) equilibrated in the same buffer. The collected peak fractions had 60–150-fold greater specific activity with DCIP (in the presence of Triton X-100) than that of the broken-cell preparation. Polyacrylamide gel electrophoresis performed on these fractions by the method of Skyring *et al.*[9] indicated the presence of at least 2 major protein contaminants. The active band was identified by immersing a duplicate gel in the DCIP–Triton X-100 assay mixture in a narrow tube and observing the appearance of a band of dye bleaching. Enzyme prepared in the way described is stable for several weeks.

Enzyme assays

Particulate dihydro-orotate dehydrogenase was assayed spectrophotometrically (Zeiss PMQ IV) in 0.1 M Tris–HCl buffer, pH 7.6, unless otherwise stated. Initial rates of oxidase activity or dihydro-orotate–DCIP-reducing activity were measured in a 3-ml reaction mixture containing 10^{-3} M dihydro-orotate (Calbiochem). The oxidase activity was measured as the rate of increase in absorbance at 290 nm ($\varepsilon_{orotate} = 6.2 \cdot 10^3$ (ref. 2). The dihydro-orotate–DCIP-reduction assay system containing, in addition, $5 \cdot 10^{-3}$ M KCN and $4 \cdot 10^{-5}$ M DCIP (British Drug House) was followed by the rate of decrease in absorbance at 600 nm ($\varepsilon_{DCIP} = 20 \cdot 10^3$). Unless otherwise stated, the concentration of Triton X-100, when used, was 0.1%. For both assays, a quantity of enzyme that would produce a change of absorbance of 0.020–0.045 per 5 min under normal conditions was used. Assays were started by the addition of substrate. When the solubilized enzyme preparation contained Triton X-100 there was a small but negligible (as far as stimulatory activity was concerned) amount of detergent carried over into the assays. Dihydro-orotate dehydrogenase activity refers to activity in the DCIP assay. Specific activity is expressed as nmoles of orotic acid formed or DCIP reduced·min^{-1}·mg protein^{-1}.

The snake venom (Boch-Light) solutions (2 mg/ml in 10 mM Tris–acetate, pH 6.5) were heated at 73–75 °C for 5 min and centrifuged at 10 000 × *g* for 10 min to remove insoluble material before use. They were used in the proportion of 1 ml/4–5 mg enzyme preparation protein in 40 mM Tris–HCl (pH 7.6)–2 mM $CaCl_2$ at 32 °C. Pure pig pancreatic phospholipase A_2 (EC 3.1.1.4) (the gift of J.-P. Abita) was used in the proportion of approx. 1:300 (weight protein).

Protein was estimated by the method of either Lowry *et al.*[10] or Yonetani[11]. Phospholipids were extracted and assayed by the method of Ames[12].

Brij 35 was from Sigma and Nonidet P42 from BDH Chemicals, Ltd.

RESULTS

Effect of Triton X-100 on dihydro-orotate dehydrogenase activities

For testing the effect of Triton X-100 on dihydro-orotate dehydrogenase, the detergent was simply added to assay mixtures in various concentrations. No preincu-

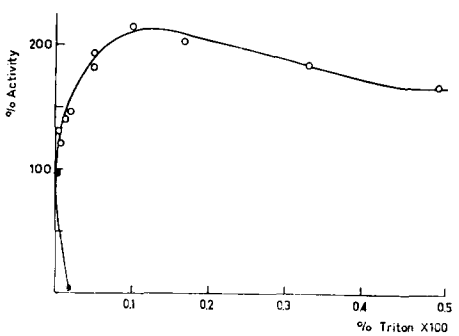

Fig. 1. Dihydro-orotate oxidase and DCIP-reducing activities as functions of Triton X-100 concentration. Dihydro-orotate oxidase activity (●—●) was measured at pH 8.3 in 100 mM Tris–HCl. DCIP-reducing activity (○—○) was measured at pH 7.0 in 100 mM potassium phosphate. Enzyme in this experiment had specific dihydro-orotate oxidase and DCIP-reducing activities of 8.06 and 5.7, respectively, under these conditions and in the absence of Triton X-100.

bation was necessary. As Fig. 1 shows, concentrations in the range of 0.01–0.02% Triton X-100 essentially eliminate dihydro-orotate oxidase activity in particulate preparations. The ability to reduce DCIP, on the other hand, is stimulated by the detergent, the maximal stimulation being attained at a detergent concentration of 0.1% regardless of the enzyme purity and protein concentration within the limits usually assayed, that is, up to 80 μg/ml. As the detergent concentration is increased further, activity with DCIP starts to fall off. Stimulation varies from 10% to approx. 3.5-fold unless the preparation had been treated with phospholipase A_2 (see below). No strict correlation of percent stimulation was found with the phospholipid content or with the ratio of phospholipid to protein although as Table I shows, an increase in purity tends to be accompanied by an increase in the percent stimulation.

TABLE I

INCREASE OF STIMULATION BY TRITON X-100 OF DIHYDRO-OROTATE DEHYDROGENASE WITH ENZYME PURIFICATION

Enzyme preparation	*Specific activity*	*Ratio activity in the presence of Triton X-100/activity in the absence of Triton X-100*
Cell-free extract	10.2	1.1
Washed particles	38	1.5
High-speed Triton X-100 supernatant	58.5	2.0
30–50 $(NH_4)_2SO_4$ fraction	241	2.0
Peak activity from agarose column	663	2–2.5

If a particle preparation is treated with Triton X-100 and then assayed in the absence of detergent, a loss of up to 50% of the pretreatment dihydro-orotate dehydrogenase activity is observed although activity in the presence of Triton X-100 is the same as if there were no pretreatment. The net result is an increase in the percent stimulation by Triton X-100.

For the sake of comparison, another nonionic detergent, Brij 35, was tested under the same conditions. This detergent has qualitatively the same effect as Triton X-100 on the dihydro-orotate oxidase and dehydrogenase activities except that 100% oxidase activity destruction and maximal DCIP-reducing activity are attained at $<$0.01% and 0.02% detergent, respectively, and there is no falling off of the latter activity with increasing detergent concentrations up to 0.6%. Moreover, stimulation of activity in the DCIP assay is 20–80% greater than that by Triton X-100.

A third nonionic detergent, Nonidet P42, was also tested and found to stimulate activity with DCIP as much as Triton X-100 but the optimal concentration was four times that of Triton X-100.

Solubilizing activity of Triton X-100

The dihydro-orotate dehydrogenase-bearing particles are insoluble and readily sedimented by centrifugation at 100 000 $\times$ *g* for 1 h. However, when a particle

preparation containing 3 mg of protein per ml is made 0.1% in Triton X-100 (optimal for stimulation), 80–100% of the activity can no longer be sedimented by centrifugation at 165 000 × *g* for 2 h. Since 20–40% of the other particulate proteins remain sedimentable some purification is attained. Isopycnic centrifugation in sucrose gradients shows that this solubilization changes the density of the activity-bearing particle from 1.17 ± 0.005 to 1.032 ± 0.002. In order to estimate the change in particle size, solubilized and non-solubilized enzyme preparations were filtered on columns of agarose beads of different exclusion limits. By this criterion, the oxidase-bearing particles have a molecular weight greater than 10^7. Filtration of the solubilized enzyme gave results that varied with the filtration solution used. Estimates of 150 000, 250 000, and 700 000 were obtained with 40 mM Tris–HCl (pH 7.6)–1 mM EDTA containing 0.4 M $(NH_4)_2SO_4$, 0.1% Triton X-100, or no addition, respectively. Fractions obtained from columns equilibrated with the $(NH_4)_2SO_4$-containing buffer were found to be concentrated by Diaflo filter XM50 but not by XM100. For this reason another estimate was made using a Sephadex G-150 column equilibrated in the salt medium. The proteins that were used to standardize the column (horse hemoglobin, ovalbumin, and bovine serum albumin) filtered as monomers in this medium. The dihydro-orotate dehydrogenase peak coming unexpectedly soon after the void volume, only a very rough estimate of 680 000 was obtained. A similar problem was encountered by Eldefrawi *et al.*[13] who suggested that their protein formed aggregates after being concentrated. This possibility is being investigated.

Soluble enzyme that had been purified through the $(NH_4)_2SO_4$ step was tested for oxidase activity and found to have barely 2% of the Triton X-100-stimulated DCIP-reducing activity (the oxidase activity is usually about one-third higher than the Triton X-100-stimulated DCIP-reducing activity in non-solubilized particles).

When Brij 35 was added to particle preparations at 0.02%, the optimal stimulatory concentration, no solubilization of dihydro-orotate dehydrogenase was observed. At 0.1% it converted about 13% of the activity but about one-third of the total protein into a non-sedimentable form. Moreover, when used with a solubilizing concentration of Triton X-100, Brij 35 reduced the percentage of activity solubilized to 64%. Thus, the two detergents interact preferentially with each other rather than with the protein to give a mixture of lower solubilizing power.

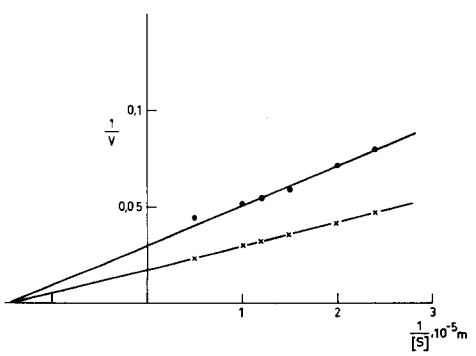

Fig. 2. Lineweaver–Burk plots of dihydro-orotate dehydrogenase activity (DCIP assay) with (×—×) and without (●—●) 0.1% Triton X-100. Standard assay conditions (0.1 M Tris–HCl buffer, pH 7.6) were used. Enzyme used was purified through the $(NH_4)_2SO_4$ step.

Effect of Triton X-100 on $K_{m,\,app}$ and inhibition by orotate

Early in this work, dihydro-orotate oxidase activity was regularly assayed at pH 8.3. The $K_{m,\,app}$ for dihydro-orotate at this pH is $2 \cdot 10^{-5}$ M. At pH 7.6, the pH used in most of these experiments, the $K_{m,\,app}$ for both oxidase and DCIP-reducing activity is $0.9 \pm 0.1 \cdot 10^{-5}$ M. If Triton X-100 is added to the assay mixtures of a particulate preparation, the Lineweaver–Burk plot (Fig. 2) indicates practically no change of $K_{m,\,app}$, although the enzyme can no longer be considered to be particulate. In other words, the change from a particulate to a soluble state in Triton X-100 is not paralleled by a change in enzyme affinity for substrate.

Dihydro-orotate dehydrogenase is inhibited by orotate, the product of its activity. As Fig. 3a indicates, the inhibition is of a mixed type. However, if the activities are measured with Triton X-100 present, the inhibition becomes competitive (Fig. 3b). This contrast is observed when the enzyme preparation used is initially soluble as well as when it is initially particulate.

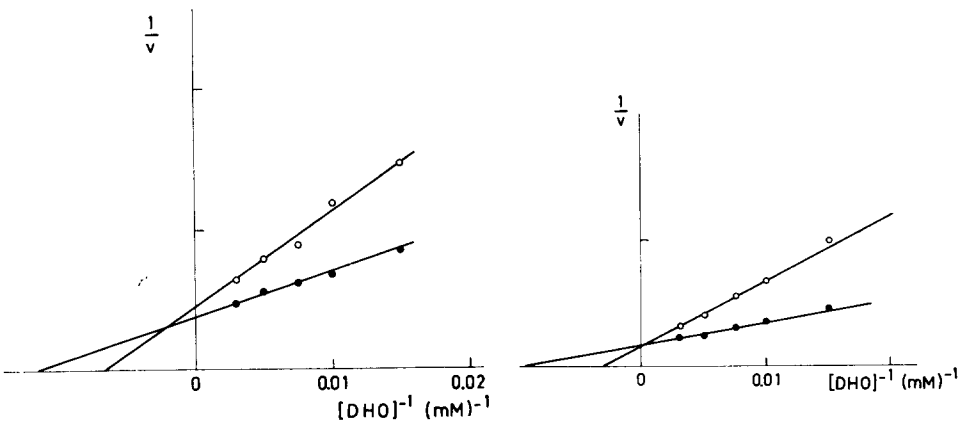

Fig. 3. Effect of Triton X-100 on the competitivity of orotate in dihydro-orotate dehydrogenase activity. (a) Plots of $1/v$ *versus* $1/S$ in the presence (○—○) and absence (●—●) of sodium orotate. (b) Same as in a except that reaction mixtures contained 0.1% Triton X-100. Concentration of orotate (DHO) was $1.67 \cdot 10^{-5}$ M. Enzyme in this experiment was a particulate preparation having specific activities of 102 and 184 in the absence and presence, respectively, of 0.1% Triton X-100. Ordinate units are the same in a and b.

Phospholipase A_2-treated enzyme

Analysis of various enzyme preparations both before and after solubilization showed that they contained a minimum of 0.1 μmole of lipid phosphorus per milligram of protein. In order to determine whether these phospholipids have a role in the catalytic activity, both particulate and soluble enzyme were treated with phospholipase A_2 from snake venoms and from pig pancreas. After a few minutes of this treatment the dihydro-orotate oxidase activity of the particulate preparation is lost. The DCIP-reducing activity drops to approx. 70% initially and then may decline to a minimum of about 10%. It was observed that as the soluble enzyme was further purified it lost less activity when treated with phospholipase A_2. This could be interpreted to mean that, since the more purified preparations contain less total phospholipid (even if the lipid/protein ratio remains constant), phospholipase A_2 treatment

TABLE II

INACTIVATION OF DIHYDRO-OROTATE DEHYDROGENASE BY PHOSPHOLIPASE A AND RESTORATION BY TRITON X-100

High-speed Triton X-100 supernatant was incubated overnight as described in Materials and Methods.

Enzyme	*Relative activity**	
	Without Triton X-100	*With Triton X-100*
Control	100	364 (100)
After treatment with *Naja* venom	42.5	388 (107)
After treatment with *Crotalus* venom	9.1	210 (58)

* Activities are related to control without Triton X-100 taken as 100. Parentheses values relate the Triton X-100 values to the Triton X-100 control taken as 100.

generates a smaller amount of inhibitory products. On the other hand, if one takes into account the fact that this phenomenon goes hand in hand with greater stimulation by Triton X-100, it could constitute a first indication of a role for phospholipids in the activity. If the partially inactivated enzyme is assayed in a Triton X-100-containing system, one observes a marked restoration of activity (Table II). In fact, if the enzyme was treated with phospholipase from *Naja naja* venom or pig pancreas it often has a higher Triton X-100-stimulated activity than the control. When the

TABLE III

STIMULATION OF TRITON X-100-SOLUBILIZED DIHYDRO-OROTATE DEHYDROGENASE OF *E. coli* BY PHOSPHATIDYLETHANOLAMINE AND DIPHOSPHATIDYLGLYCEROL

Expt. 1: 2.2 μg enzyme preparation protein was pre-mixed with the indicated amount of phospholipid in 40 μl in 40 mM Tris–HCl (pH 7.6)–200 mM $(NH_4)_2SO_4$–0.5 mM EDTA–20% ethanol.* Expt. 2: 1.5 μg phospholipase-treated enzyme preparation protein was pre-mixed in 5 μl in 40 mM Tris–HCl (pH 7.6)–0.5 mM EDTA–14% methanol* with the indicated amount of phospholipid.

Phospholipid (nmoles)	*Relative activity*	
	Without Triton X-100	*With Triton X-100*
Expt 1		
None	100	315
Phosphatidylethanolamine (2.4)	113	312
Phosphatidyl ethanolamine (24)	151	312
Diphosphatidylglycerol (1.8)	120	315
Diphosphatidylglycerol (18)	189	322
Phosphatidylethanolamine (24) *plus* diphosphatidylglycerol (1.8)	183	308
Expt 2		
None	100	358
Phosphatidylethanolamine (2)	120	319
Diphosphatidylglycerol (2.35)	234	348

* This concentration of solvent did not affect the control.

Crotalus treatment was allowed to go to completion, Triton X-100 restored only about half the control Triton X-100-stimulated activity. The molecular weight of the phospholipase-treated enzyme was roughly estimated as 390 000 on the same Sephadex G-150 column used for the untreated enzyme.

In order to determine whether the loss of dihydro-orotate dehydrogenase activity was due to the loss of phospholipids that might be necessary for maximum activity, or to inhibition by degradation products, the effects of various phospholipids and their degradation products on the enzymatic activity were tested. Table III shows the effect of phosphatidylethanolamine and diphosphatidylglycerol on dihydro-orotate dehydrogenase in the presence and absence of Triton X-100. Diphosphatidylglycerol is considerably more effective than phosphatidylethanolamine in stimulating the activity. Under these conditions neither stimulates as much as the detergent. Part of the reason for this may be that all Triton X-100-solubilized preparations contain some detergent despite prolonged dialysis and filtration through an agarose column. This is indicated by the easy foaming and the ultraviolet spectra of these preparations. Triton X-100, which probably adheres to enzyme sites that would have an affinity for phospholipids, may diminish association of added phospholipids with protein by forming mixed micelles with them. It may also be noted that activity measured in the presence of the detergent remains essentially constant with varying

TABLE IV

EFFECT OF PHOSPHOLIPASE A_2-TREATED PHOSPHATIDYLETHANOLAMINE ON TRITON X-100 SOLUBILIZED DIHYDRO-OROTATE DEHYDROGENASE OF *E. coli*

Conditions were those described in Table III, Expt. 1, except that the phospholipid was preincubated with pancreatic phospholipase and the hydrolysis stopped by adding EDTA to 5 mM.

Phospholipid added (nmoles)	*Relative activity*	
	Without Triton X-100	*With Triton X-100*
0	100	355
1.2	90	364
12	61.3	332

amounts of added phospholipid. Equivalent amounts of total *E. coli* phospholipids extracted from exponentially growing and stationary-phase cells stimulated these preparations only up to 25%. This could be caused by the presence of an inhibitory product.

If phosphatidylethanolamine is submitted to phospholipase A_2 action before incubation with enzyme, it is inhibitory (Table IV) especially at higher concentrations. The response of dihydro-orotate dehydrogenase to phospholipase-treated diphosphatidylglycerol is more complicated (Fig. 4). At lower concentrations phospholipase-treated diphosphatidylglycerol is somewhat stimulatory whereas at higher concentrations it becomes inhibitory. Throughout the concentration range tested Triton X-100 restores a little more than 100% activity.

Since the degradation products of phospholipase-treated phosphatidylethanolamine, which comprises the bulk of the phospholipids of *E. coli*, consists of lysophos-

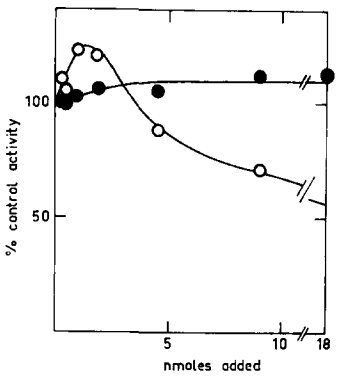

Fig. 4. Dihydro-orotate dehydrogenase activity as a function of concentration of phospholipase A_2-treated diphosphatidylglycerol. Conditions were those described in Table IV. Relative activity in the presence (●—●) and absence (○—○) of 0.1% Triton X-100.

phatidylethanolamine and fatty acid, a large percentage of which is unsaturated[14], it was of interest to determine the individual effects of these compounds. As Table V shows, oleic acid inhibits dihydro-orotate dehydrogenase to about the same extent as did the phospholipase-treated phosphatidylethanolamine. Lysophosphatidylethanolamine, on the other hand, has a small but definite stimulatory effect which is reflected in the slight relief it gives from the oleic acid-mediated inhibition when both degradation products are present.

The higher concentrations of lipid used in these experiments represent large excesses with respect to the lipid/protein ratios usually found in particulate preparations (0.15–0.50 μM lipid/mg protein). However, such concentrations may not exceed those of the environment of individual proteins in the complex(es). In the case of dihydro-orotate dehydrogenase, the difficulty of removing Triton X-100 suggests that this enzyme is a lipophilic protein[15]. In order to eliminate the fatty acid contribution to the overall activity loss that occurs during phospholipase treatment, an excess of delipidated bovine serum albumin (which effectively abolishes inhibition by added

TABLE V

EFFECT OF LYSOPHOSPHATIDYLETHANOLAMINE AND OLEIC ACID ON TRITON X-100 SOLUBILIZED DIHYDRO-OROTATE DEHYDROGENASE OF *E. coli*

Conditions were those described in Table III, Expt. 1, except that there was no ethanol in the premixture.

Lipid added (nmoles)	*Relative activity*
None	100
Lysophosphatidylethanolamine (2)	111
Lysophosphatidylethanolamine (20)	124
Oleic acid (2)	83
Oleic acid (20)	61
Lysophosphatidylethanolamine (2) + oleic acid (2)	93
Lysophosphatidylethanolamine (20) + oleic acid (20)	72

oleic acid) was added to a cell-free extract subjected to phospholipase. This lipid-binding protein, at concentrations of 0.2–0.3 mg/ml, also stimulates dihydro-orotate dehydrogenase activity (up to 32% in cell-free extract, 100% in the partially purified soluble preparation used in these experiments). It was found that phospholipase treatment still caused 47% inhibition with respect to the phospholipase-less control. This probably represents a minimum inhibition because with the removal of fatty acids the observed activity would be lysophospholipid-stimulated.

DISCUSSION

Despite its potential interest as a model membrane-bound enzyme and as a link between respiratory and biosynthetic systems in *E. coli*, dihydro-orotate dehydrogenase has received relatively little attention. Low activity levels[16] and difficulty in stabilizing the solubilized enzyme have been reported[17]. The former problem can be attenuated partly by the use of derepressible strains, and partly by Triton-activation of the enzyme; the latter problem seems to be resolvable by the use of Triton X-100 as the solubilizing agent. Although a certain loss of activity is observed when Triton X-100 is removed, the activity is "recovered" by simply including Triton X-100 in the assay mixture.

The activity lost by phospholipase A_2 treatment may or may not be completely restored by the detergent. Less restoration is observed if the source of phospholipase A used is *Crotalus adamanteus* venom which does not attack diphosphatidylglycerol[18]. Under these conditions the only phospholipid degradation products formed are those formed from phosphatidylethanolamine (and possibly from phosphatidylglycerol although much of this phospholipid would be converted to diphosphatidylglycerol in stationary-phase cells[19]). Since the detergent seems to abolish the effects of these products, the reason for this behavior is not clear.

At concentrations lower than that required to solubilize dihydro-orotate dehydrogenase, Triton X-100 interrupts the electron transport chain. Whether this initial effect takes place at the level of a lipid as suggested by Kerr and Miller[3] or at the site of a hydrophobic protein–protein interaction in not clear.

Under the conditions used, Triton X-100 does not solubilize all of the particulate proteins; this is also true of Brij 35 but the latter seems to be antiselective with respect to dihydro-orotate dehydrogenase. It may be that by using a proper sequence of these and other detergents it will be possible to effect a useful fractionation of membrane proteins.

The results presented here are consistent with the idea that the variable amount of stimulation by Triton X-100 of DCIP-reducing activity is due to the variable loss or modification of the normal lipid environment of the enzyme from the moment the bacterial envelope is broken and continuing thoughout purification steps. Triton X-100 as well as Brij 35 and bovine serum albumin in some measure substitute for the lost lipid to restore activity. Since Triton X-100 modifies the inhibition by orotic acid, the product of dihydroorotic acid oxidation, from a mixed to a competitive type, it can be imagined that the detergent quickens product release, thereby increasing substrate turnover.

Dihydro-orotate dehydrogenase of *E. coli* has been solubilized using deoxycholate[3]. The deoxycholate-solubilized enzyme complex is undoubtedly larger and

contains more lipid than the one described here since it is excluded from Sephadex G-200 and separates by flotation in 30% $(NH_4)_2SO_4$. Moreover, that preparation seems to retain at least 17% of its oxidase activity. According to Taylor *et al.*[17] deoxycholate-solubilized dihydro-orotate dehydrogenase is very unstable. It is possible that the smaller micelles formed by the anionic detergent[13] are more readily removed than Triton X-100 and hence leave the enzyme more susceptible to denaturation by aggregation or by conformational changes.

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