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DIHYDRO-OROTATE OXIDASE OF *ESCHERICHIA COLI* K12: PURIFICATION, PROPERTIES, AND RELATION TO THE CYTOPLASMIC MEMBRANE

D. KARIBIAN* and P. COUCHOUD

Laboratoire Chimie Bacterienne, Centre National de la Recherche Scientifique, 31, chemin J. Aiguier, 13, Marseille (France)

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SUMMARY

The membrane-bound, biosynthetic dihydro-orotate oxidase of *Escherichia coli* K12 has been purified 400–800-fold. The molecular weight of the enzyme was found to be about 67 000. The spectrum of enzyme solutions in the visible light range shows no indication that flavins are present.

The solubilized enzyme is stimulated by ammonium sulfate. When added together with Triton X-100, another stimulator of the *E. coli* enzyme, ammonium sulfate stimulates 58% more than would be expected for an additive effect.

The particulate enzyme is stabilized to heating at 60 °C by its substrate and by orotate, the product of its reaction.

The nature of dihydro-orotate oxidase binding on the membrane is inferred from:

(1) Solubilization data. Dihydro-orotate oxidase can be solubilized completely by Triton X-100 or by phospholipase A_2 treatment followed by a shift of pH from 7.6 to 8.4. Only partial solubilization is attained by alkaline pH alone, or by phospholipase treatment plus high salt concentration or bovine serum albumin. Phospholipase-solubilized and purified enzyme tends to aggregate more than Triton X-100-solubilized enzyme.

(2) Arrhenius plots which are biphasic (transition at 19 °C) for particulate or Triton X-100-solubilized enzyme, straight for phospholipase-solubilized enzyme, and biphasic for the latter when activities are measured in the presence of Triton X-100.

(3) Experiments testing enzyme accessibility to trypsin action. These suggest that dihydro-orotate oxidase is located on the inner side of the cytoplasmic membrane.

INTRODUCTION

Biosynthetic dihydro-orotate oxidase (EC 1.3.3.1) which is membrane-bound in *Escherichia coli* [1], *Pseudomonas* sp. [2, 3], and *Staphylococcus aureus* [4] is of

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

* Present address: Centre de Biochimie et de Biologie Moleculaire, 31, chemin J. Aiguier, 13, Marseille, France.

interest as a link between the cell's respiratory system and the pathway of pyrimidine synthesis. In *E. coli*, this enzyme is repressible by pyrimidine end products [5] and its activity in the cell depends on a functional electron transport system [1, 6] to accept the reducing equivalents produced during catalysis.

The biosynthetic enzyme is not to be confused with the inducible, cytoplasmic, pyridine nucleotide-linked enzyme of *Zymobacterium oroticum* [7] and other bacteria [2, 3, 8]. The latter enzyme seems to function primarily as an orotate degradative enzyme and has not been found in *E. coli* although certain bacteria do contain both the biosynthetic and the degradative enzyme [2, 3, 9]. Taylor et al. [9] have studied what seems to be an intermediate type of dihydro-orotate oxidase in *Lactobacillus bulgaricus* where the enzyme is cytoplasmic, biosynthetic, and a flavoprotein about half the size of the *Zymobacterium* enzyme. It will not be possible to discover an evolutionary relationship amongst these different types of dihydro-orotate oxidase until they are each well characterized. The dihydro-orotate oxidase of *E. coli* has been partially purified after solubilization by the detergents deoxycholate [2, 6] and Triton X-100 [10]. In order to characterize the enzyme we improved the purification system using the non-ionic detergent as solubilizing agent since it has the advantage of activating and stabilizing the enzyme. The improved method includes: (1) more selective solubilization conditions, (2) more reproducible conditions for molecular sieve filtration, and (3) the addition of a chromatography step. The enzyme preparations obtained had specific activities 400–800 times those of crude extracts made from derepressed cells. The purification and several properties of the enzyme are reported here.

Since earlier work had shown that phospholipids play a role in dihydro-orotate oxidase activity [10], we also examined the relation of the enzyme to the membrane, i.e., the nature and tightness of their association. This was done by investigating (1) the treatments necessary for solubilizing the enzyme, (2) the extent of enzyme penetration into the bilayer, and (3) the possible influence of lipids on activation energy.

MATERIALS AND METHODS

The *E. coli* K12 strain used was a Pyr E^- (lacking orotidine-5′-phosphate pyrophosphorylase (EC 2.4.2.10)) derived by transduction from *E. coli* strain AT1243 [10]. The *Salmonella typhimurium* Pyr H700 (formerly HD-58 [11]) having a partial loss of uridine-5′-phosphate kinase was a gift of J. C. Gerhart. Unless otherwise stated, the cells were grown as previously described [10] on minimal-casamino acids–glucose medium containing 8 μg/ml uracil, derepressed for two h after uracil depletion, and harvested. The cells were suspended in Tris–HCl, 40 mM, pH 7.6 (2 g wet weight/10 ml buffer), and broken in a French press (Aminco) at 4 tons/inch^2. The extract was treated with deoxyribonuclease and ribonuclease 1 μg/ml each, and then centrifuged at 100 000 $\times$ *g* for 1 h or 60 000 $\times$ *g* for 2 h. The pellet was washed 2–3 times in one-fifth of the original volume of the same buffer and then resuspended to a protein concentration of 50 mg/ml.

The enzyme assays were carried out as before [10]. Briefly, dihydro-orotate oxidase was assayed spectrophotometrically either (1) by dichlorophenolindophenol (DCIP) reduction using $0.42 \cdot 10^{-4}$ M DCIP ($\varepsilon_{600\ \mathrm{nm}} = 20$) at pH 7.6 (Tris–HCl 100 mM), with 5 mM KCN to suppress electron flow toward O_2 when particulate enzyme

was used, or (2) by oxygen uptake, the appearance of orotate being followed at 290 nm ($\varepsilon_{290\ nm} = 6.2$ [12]). Only particulate enzyme has this activity. In both assay systems the reaction was started by adding 1 mM sodium dihydro-orotate oxidase.

Protein was assayed by the method of Lowry et al. [13] using bovine serum albumin as standard. Agarose columns were prepared (53 cm × 2.6 cm) with Biogel A-1.5 200–400 mesh suspended in the indicated buffer (see Results and Discussion), packed by gravity, and washed with a liter of the same buffer before use. Columns were run at room temperature. Flow rate 25 ml/h. Fraction volumes were usually about 3.6 ml. Polyacrylamide gel electrophoresis was run as indicated by Canalco with the modification of Skyring et al. [14], i.e., with 0.05% and 0.1% Triton X-100 in the separating and stacking gels, respectively. Spectra were taken with a Cary 14.

Snake venom was heated and centrifuged before use as described [10] but was used in the proportion 0.2 ml/100 mg particulate protein in 5 ml of 40 mM Tris–HCl (pH 7.6)–mM $CaCl_2$. Lipids were extracted by the method of Bligh and Dyer [15], chromatographed on thin layers (Merck TLC plates Silica gel 60, 0.25 mm), developed in chloroform : methanol : acetic acid (65:25:8, by vol.). The spots revealed by iodine vapors were scraped off, eluted with chloroform–methanol (1:1, by vol.), and assayed for phosphate by the method of Ames [16].

Trypsin, trypsin inhibitor, and defatted bovine serum albumin were from Sigma Chemicals.

Data for Arrhenius plots were calculated from measurements made using a Cary 14 spectrophotometer with a temperature control and a 0–0.1 scale. The temperature of fluid leaving the cuvette compartment was monitored by thermocouple connection with a Meci speedomax H indicator and regulated by a Haake circulating bath.

RESULTS AND DISCUSSION

Earlier work had shown that the nonionic detergent Triton X-100 (*iso* octylphenoxypolyethoxyethanol having 9–10 ethoxy groups) solubilizes dihydro-orotate oxidase but that the solubilization gives little purification, and subsequent efforts to purify the solubilized enzyme proved to be difficult. We therefore tried to achieve a greater degree of specificity of solubilization and hence a better purification by varying the pH, the ionic strength, and the presence or absence of Mg^{2+}. Schnaitman [17] has shown that in the presence of Mg^{2+}, Triton X-100 solubilizes the cytoplasmic (inner) membrane of *E. coli* leaving the outer membrane insoluble. Other workers have shown that pH and ionic strength modify the solubilizing power of Triton X-100. We have, tested the degree of solubilization in the pH range 7–8.75 with buffer molarity varying from 0.004 to 0.2 with the addition of either EDTA or 5 mM $MgCl_2$. The results presented in Table I show that, in general, the solubilization of dihydro-orotate oxidase is maximized and the specificity of solubilization is optimum at low ionic strength pH 7, in the presence of magnesium. The highest activities represent 100% recovery. The specific activities attained in this experiment with Mg^{2+} present are about twice those obtained with EDTA and represent a six-fold increase over that of the particulate enzyme.

The supernatant can be concentrated by filtration using a Diaflo XM50 membrane and then fractionated with ammonium sulfate. Enzyme precipitating between

TABLE I

SOLUBILIZATION OF DIHYDRO-OROTATE OXIDASE BY TRITON X-100 AS A FUNCTION OF pH, IONIC STRENGTH, AND THE PRESENCE OR ABSENCE OF AVAILABLE Mg^{2+}

A washed particle preparation was divided into nine pairs which were diluted 10-fold with buffer of appropriate molarity and pH (the pH values listed are measured values). EDTA (mM) was added to one member of each pair and $MgCl_2$, 5 mM, was added to the other. Triton X-100, 0.1%, was added to all, and the suspensions were centrifuged at 150 000 × *g* for 1 h. The supernatants were assayed for protein and for DCIP reductase activity in the presence of Triton X-100.

Buffer	pH	Molarity	Activity in presence of Triton		Specific activity	
			EDTA	Mg^{2+}	EDTA	Mg^{2+}
Sodium, potassium phosphate	7	0.004	97	118	44.4	98.4
	7	0.02	95	126	46.4	93.6
	7	0.2	107	121	44.4	56
Tris–HCl	7	0.008	103	80	41.8	84
	8.1	0.008	95	89	30	78
	7.5	0.024	107	84	40.4	70.7
	8.5	0.024	89	83	25.7	59
	7.8	0.2	102	89	29.2	49.5
	8.7	0.2	91	92	22.2	38.6

40 and 50% has the highest specific activity. The precipitate is resuspended in 10 mM Tris, pH 8.4, containing 10 mg% Triton X-100 and dialyzed against the same buffer containing 0.6 M ammonium sulfate. The dialyzate is centrifuged at 150 000 × *g* for 1 h and then the protein concentration adjusted to 2.5 to 3 mg/ml to control the enzyme's tendency to aggregate before loading on an agarose column equilibrated with the above Tris–high salt buffer. At this protein concentration the enzyme is retarded enough to achieve a good separation from many of the contaminants. Activity comes off at about 1.8 void volumes in a broad beak. The best fractions are collected and concentrated on a Diaflo XM 50 filter. This is followed by chromatography on a DEAE-cellulose column equilibrated with sodium–potassium phosphate (50 mM, pH 6.8)–ammonium sulfate (0.1 M). The column is washed with several volumes of the same buffer. Under these conditions, the enzyme does not adhere to the column. The peak activity comes off at about 2 column volumes of buffer. The total activity yield of this column is 50–60%, only part of which is well-separated from a yellow contaminant that precedes it. The purification procedure presented in Table II is representative. The enzyme is stable in solution at 2–25 °C. The best preparation obtained had an 840-fold purification. On disc gel electrophoresis a typical preparation appears to be nearly homogeneous (Fig. 1). The R_F of the latter with respect to bromphenol blue is 0.18. Less pure preparations migrated somewhat faster (R_F = 0.25–0.3). Skyring et al. [14] using a crude extract report an R_F still greater —0.38. This variation is undoubtedly caused by the increased delipidation that occurs with purification and that is accompanied by a greater tendancy to aggregate as well as lower activity in the absence of Triton X-100.

The spectrum of a purified preparation containing 193 μg protein/ml was taken in the ultraviolet and visible regions (Fig. 2). The asymmetric peak at 274 nm indicates

TABLE II

PURIFICATION OF DIHYDRO-OROTATE OXIDASE OF *E. COLI*

Preparation	Activity units (+ Triton) (μm DCIP/min)	Specific activity (− Triton) (nmoles/mg per min)	Specific activity (+ Triton) (nmoles/mg per min)	Fold purification
Crude extract	141.5	5.7	7.5	1
Washed particles	83.5	29.2	63	8.4
Solubilized enzyme, 150 000 × *g* supernatant	102.0	157.2	414	55
Precipitate 40–50% saturation $(NH_4)_2SO_4$	38.2	271	1020	136
Agarose column peak	18.1	—	2730	364
DEAE-cellulose column best fractions	6.8	—	3630	484

that there is enough Triton X-100 present (a solution of 0.01% Triton X-100 in the same buffer has $\varepsilon_{274}^{max} = 0.213$) to shift the maximum from the usual protein peak. Triton X-100 was absent in the final dialysis and chromatography. There is no indication of flavin absorption at about 450 nm. This corroborates the work of Kerr and

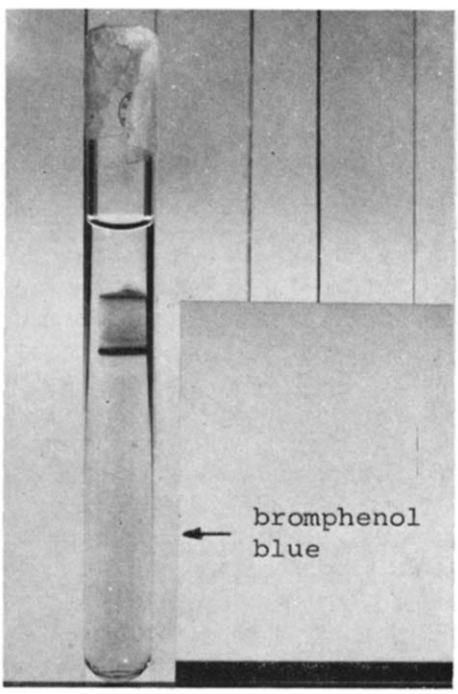

Fig. 1. Disc gel electrophoresis of dihydro-orotate oxidase. The polyacrylamide gel (7%) contained 0.05% Triton X-100. Protein was stained with Coomassie blue. Coincidence of the major band with the active band was verified by the bleaching of DCIP in an unstained identical gel. The enzyme was an active fraction from the DEAE-cellulose column.

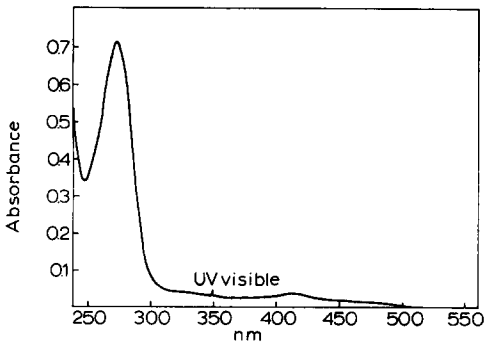

Fig. 2. Spectrum of dihydro-orotate oxidase in the ultraviolet and visible regions. Enzyme is an active fraction from the DEAE-cellulose column.

Miller [6] who, although finding FMN in semi-purified preparations made from *E. coli* B, were not able to detect flavin fluorescence. The membrane-bound dihydro-orotate oxidase of *E. coli* differs in this respect, therefore, from the cytoplasmic dihydro-orotate oxidases, whether biosynthetic or degradative.

Assuming the best preparations to be pure and a molecular weight of about 67 000 (see below), we have estimated from their activity that the turnover number is about 284 moles of DCIP reduced/mole enzyme per min in presence of Triton. It is hard to compare this with the turnover numbers of other dihydro-orotate oxidases even when reported for DCIP as the electron acceptor, because the relation of DCIP-reducing activity to oxidase or ferricyanide-reducing activity seems to vary greatly from one bacterial strain to another. In any case, at this catalytic rate, and with normal constitutive levels of enzyme, the cells would be able to synthesize more than enough pyrimidine for its needs if this step were limiting. K12 strains do, in fact, overproduce pyrimidines [18, 19].

Molecular weight determination

Earlier efforts to determine the molecular weight of dihydro-orotate oxidase of *E. coli* K12 gave inconclusive results because of the enzyme's tendency to aggregate [10]. Since this tendency is partly a function of protein concentration we have used it together with optimal conditions of ionic strength and pH to resolve the problem. As before, a standardized agarose column equilibrated with 40 mM Tris–HCl (pH 7.6)–0.6 M $(NH)_2SO_4$–5 mM EDTA–0.1 % Triton was used. Two estimates were made with each of two enzyme preparations (both having specific activities of about 540) of identical starting volume but varying protein concentration. These estimates were plotted as a function of total protein loaded on the column (Fig. 3). Extrapolation of the plot to zero protein gives a molecular weight of about 67 000.

Stimulation by ammonium sulfate

In the course of this work, we observed that ammonium sulfate stimulates solubilized dihydro-orotate oxidase activity, but has little effect on the particulate enzyme. The optimum concentration is about 0.6 M and, depending on the enzyme preparation, maximum stimulation varies from 33 to about 120%. At equivalent ionic strength, the acetate and phosphate salts are equally effective, but sodium

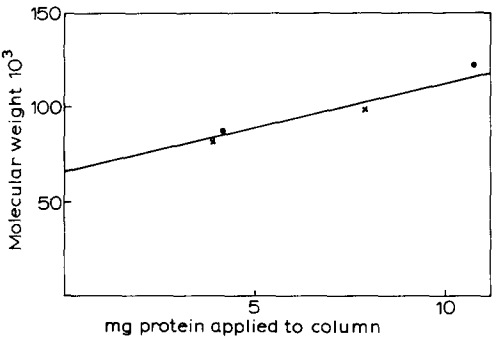

Fig. 3. Molecular weight determination of dihydro-orotate oxidase. Two preparations (3.6 ml) of similar specific activity were passed over an agarose column twice each under identical conditions except that the protein concentration of the sample was varied. The column was standardized with ovalbumin, chymotrypsinogen, and bovine serum albumin, and equilibrated with 40 mM Tris–HCl (pH 7.6)–0.6 M ammonium sulfate–5 mM EDTA–0.1% Triton X-100.

sulfate is 63% as good. When ammonium sulfate is used with Triton X-100 (0.1%) the stimulation is not additive but moderately synergistic (Table III). Ammonium sulfate also stimulates the biosynthetic dihydro-orotate oxidase of *L. bulgaricus* [9]. If the salt stimulates the *E. coli* enzyme by antagonizing the formation of less active enzyme polymers (as seems likely) it is probable that the effect on the *Lactobacillus* enzyme is not the same since polymerization would not be a problem with a cytoplasmic enzyme.

TABLE III

SYNERGY OF STIMULATION BY AMMONIUM SULFATE OR PHOSPHATE WITH THAT BY TRITON X-100

Solubilized dihydro-orotate oxidase was used. Effectors were added directly to the reaction mixture.

Effectors	Activity
None	35
$(NH_4)_2SO_4$, 0.4 M	46
Triton X-100, 0.1%	100
Triton X-100, 0.1%, plus $(NH_4)_2SO_4$, 0.4 M	175 (111)*
$(NH_4)_2HPO_4$, 0.4 M	49
Triton X-100, 0.1%, plus $(NH_4)_2HPO_4$, 0.4 M	183 (114)*

* Figures in parentheses represent activities expected for an additive effect.

Thermostability

Another way of characterizing the enzyme was to test its stability to heating at 60 °C. Since solubilized preparations contained detergent which might cause aberrations, the experiments were limited to particulate preparations. Variations of the protein concentration of the heated sample from 90 to 900 μg/ml did not affect the stability. The half-life of dihydro-orotate oxidase was found to vary from 3 to 17 min depending on the preparation although the half-life tended to be shorter after washing the particles. Fig. 4 shows the shape of the inactivation curve. The enzyme substrate and the product of its activity, orotate, were found to protect dihydro-orotate oxidase

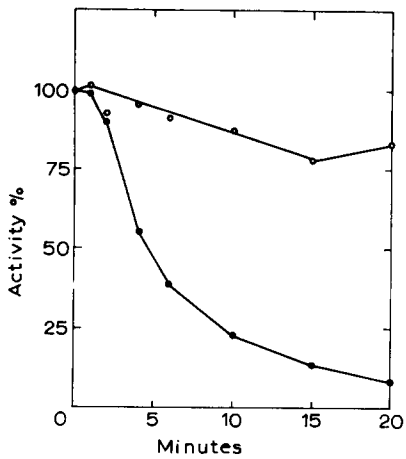

Fig. 4. Heat inactivation of dihydro-orotate oxidase and protection by orotate 1 mM. The particulate enzyme preparation in 40 mM Tris–HCl (pH 7.6)–0.1 M KCl was heated at 60 °C and samples removed at intervals into assay mixtures. ●—●, control; ○—○, with orotate.

equally well against inactivation. The curve of inactivation of dihydro-orotate oxidase coincides with that of the primary dehydrogenase (Fig. 5). This suggests that dehydrogenase is at least as heat-sensitive as any other element of the oxidase system.

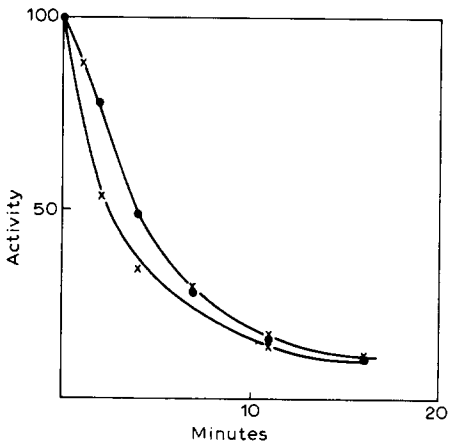

Fig. 5. Thermal inactivation curves of dihydro-orotate oxidase measured as DCIP reduction or as dihydro-orotate oxidase. Enzyme is a particulate preparation (600 μg/ml) in 40 mM Tris–HCl (pH 7.6)–0.1 M KCl, prepared from cells grown on minimal medium at 32 °C. ●—●, oxidase; ×—×, DCIP reduction.

Relation of dihydro-orotate oxidase to the membrane

The bulk (90–95%) of dihydro-orotate oxidase activity can be sedimented at 100 000 × *g* for 1 h. The addition of EDTA to the system has no effect on this property. This suggests that the enzyme is not held by divalent cations. Since phospholipase A_2 has been shown to liberate at least two other membrane enzymes [20, 21] we

undertook to solubilize the enzyme by this method. Washed membrane particles were treated with phospholipase A_2 of *Naja naja* venom for 2 h. During this time, dihydro-orotate oxidase activity (measured as DCIP-reducing activity in the absence of Triton X-100) decreases before levelling off. Excess EDTA was added [22] and identical samples of the hydrolysate were diluted 4-fold with buffer pH 7.6 or pH 8.45 and centrifuged. Supernatant activities were compared with activities of noncentrifuged preparations. Table IV shows that at pH 7.6, 71% of the activity sediments, whereas at pH 8.4 only 12% sediments. This is indicated better by comparing the Triton X-100-stimulated activities because these activities are not influenced by phospholipids and lysophospholipids (stimulation) or by fatty acids (inhibition). The above solubilization is selective, giving an enzyme preparation of 4–5-fold increased specific activity.

TABLE IV

SOLUBILIZATION OF DIHYDRO-OROTATE OXIDASE BY PHOSPHOLIPASE A_2 OF SNAKE VENOM

Three portions of particulate enzyme were incubated 2 h with or without snake venom (see Materials and Methods). The suspensions were then assayed for DCIP-reducing activity with and without Triton X-100, then divided into two parts. One part was diluted 1:4 in 40 mM Tris–HCl (pH 7.6)–1 mM EDTA and the other in Tris–HCl (pH 8.45)–EDTA. All were centrifuged first at 15 000 × *g* for 15 min, then at 100 000 × *g* for 60 min, and re-assayed as before.

Enzyme preparation	Relative activity			
	Control		*Naja*-treated	
	− Triton X-100	+ Triton X-100	− Triton X-100	+ Triton X-100
Incubation mixture	100	132	47	120
15 000 × *g* (pH 7.6) supernatant	6	11	9	35
15 000 × *g* (pH 8.4) supernatant	12	33	23	106
100 000 × *g* (pH 8.4) supernatant	18	26	17	106 (88)*

* Figure in parentheses represents activity relative to the uncentrifuged preparation.

Factors other than pH adjustment, i.e. high ionic strength (0.4 M ammonium sulfate) or excess bovine serum albumin gave only 45% solubilization of the phospholipase-treated enzyme. This suggests that at pH 7.6 the continued insolubility of the phospholipase-treated enzyme does not result only from enzyme polymerization since 0.4 M ammonium sulfate diminishes this tendency, or from enzyme adherance to fatty acid micelles since the latter can be sequestered by albumin.

These results lead us to think that, after phospholipase action on the membrane, dihydro-orotate oxidase remains attached by an ionic bond to some residual membrane structure. This bond would be broken by the deprotonation of some group having a pK of about 8. Dihydro-orotate oxidase is one of only a few enzymes that have been dissociated from the membrane by phospholipase treatment. Our results suggest that many more membrane-bound enzymes could be released this way given the proper ionic conditions.

Next, we determined how much activity could be solubilized by a shift of pH alone.

Acid pH values were of no interest because at pH values between 5 and 6 the particulate enzyme flocculates. Treatment at alkaline pH values for a short period at room temperature increased the fraction of non-sedimentable enzyme (Fig. 6). The maximum solubilization was reached at pH 10.6 with 47.5% of the control (pH 7.6) Triton-X-100-stimulated activity remaining in the supernatant. The non-stimulated activity amounted to 20% of the control. This just means that the solubilized enzyme depends more on Triton X-100 than the particulate enzyme. Incubation at 32 °C for 1.5 h at alkaline pH values before centrifuging does not increase the amount of solubilization. The best increase in specific activity attained by alkalinity was 2.5-fold.

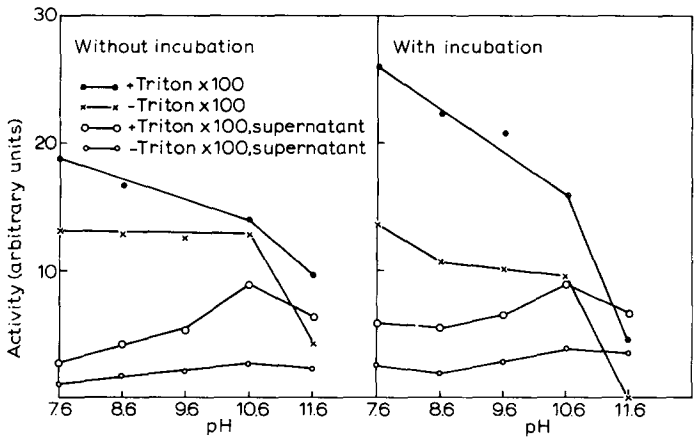

Fig. 6. Solubilization of dihydro-orotate oxidase by treatment at alkaline pH. Portions of particulate enzyme (3 mg protein/ml in 40 mM Tris–HCl –1 mM EDTA were brought to the pH indicated by addition of 1 M NaOH. Each portion was immediately sampled for assay with and without Triton X-100, then half of each portion was centrifuged at 21 °C at 10 000 × *g* for 10 min and the supernatant recentrifuged at 100 000 × *g* for 1 h. This supernatant was chilled and assayed. The other half was incubated at 32 °C for 1.5 h and centrifuged and assayed as above.

Supernatants contained 5–16 nmoles of phosphatidylethanolamine/mg protein, i.e., about one twentieth of that found in Triton X-100-solubilized preparations after fractionation with ammonium sulfate.

Alkaline pH does therefore favor solubilization of dihydro-orotate oxidase and with some selectivity although it does not solubilize all of it.

Purification of phospholipase-solubilized dihydro-orotate oxidase

The phospholipase-solubilized enzyme can be partially purified using some of the methods just described for the Triton-solubilized enzyme. However, the ammonium sulfate fractionation is performed at pH 8.4, a necessary condition also for obtaining good yields (85–95%) in the agarose filtration step. A 150-fold purification was attained. After this step the enzyme is difficult to purify further without the use of detergent. For example, concentrating it is slow because it tends to clog the membrane, and we have not been able to elute it from DEAE-cellulose without Triton

X-100. Therefore, for those whose interest in a phospholipase-solubilized enzyme is to obtain a pure membrane enzyme without using detergent, it will be necessary to test other methods of purification.

Influence of lipids on the energy of activation

Many membrane-bound enzymes display broken or discontinuous Arrhenius plots. This phenomenon seems to be typical of enzymes that are directly influenced by membrane lipid and has been attributed to a phase change in the lipid hydrocarbon chains [23, 25]. In the case of dihydro-orotate oxidase of *E. coli*, the Arrhenius plot (Fig. 7a) shows such a break at about 19 °C (the cells were grown at 37 °C). The activation energies above and below the break were calculated to be 9900 and 16 800 cal/mole, respectively. The Arrhenius plot obtained using an extract of *S. typhimurium* grown at 32 °C also has a break at 19 °C (Fig. 7b) and the slopes are similar to those of the *E. coli* enzyme.

It has been reported that non-ionic as well as anionic detergents abolish the discontinuities in the Arrhenius plots of mitochondrial respiratory enzymes [23]. We therefore determined the effect of Triton X-100 in our system. As Fig. 7b shows, addition of Triton X-100 to the reaction mixture does not abolish the break. The slopes are essentially unchanged. If the preparation is solubilized and centrifuged before making the measurements, the Arrhenius plot of activities in Triton X-100 is still biphasic but the break is at 15–16 °C; the slopes are not appreciably altered.

Treatment of the enzyme with phospholipase A_2 of cobra venom leaves no detectable unhydrolyzed phospholipid. Enzyme that was thus treated, solubilized, and partly purified gave a straight, unbroken Arrhenius plot (Fig. 7c). Its activation energy is 6000 cal/mole which is lower than either of the two activation energies of the non-treated enzyme. Phospholipase-treated dihydro-orotate oxidase is therefore less affected by temperature changes than the untreated enzyme at temperatures above the transition. One can interpret these results to mean that Triton X-100 either does not seriously perturb essential phospholipids or that it binds enzyme in a way similar to phospholipids. Phospholipase, on the other hand, denudes the enzyme.

To pursue the Triton effect a little further, we made another Arrhenius plot from activities of the phospholipase-treated enzyme measured in the presence of the detergent. Fig. 7d shows that it is again biphasic. This supports the idea that Triton X-100 mimics phospholipids in its action on the enzyme and that the Triton X-100-stimulated activity gives a good assay of the enzyme.

Localization of dihydro-orotate oxidase in the membrane

Until now, most of our experiments indicate that dihydro-orotate oxidase is at a water–hydrocarbon interface. We were therefore interested in determining how far the enzyme extends into the bilayer. To acquire this kind of information one method is to determine the accessibility of the enzyme to proteases or to chemicals that react with various surface groups. Steck et al. [26] compared the sensitivities of various erythrocyte-membrane enzymatic activities to trypsin action using inside-out and outside-out vesicles. In a similar vein, Négrel et al. [27] have correlated sulfhydryl titration and enzyme inactivation under various conditions, with enzyme accessibility.

We have compared the trypsin sensitivity of dihydro-orotate oxidase activity (assayed both as DCIP-reducing activity and as dihydro-orotate oxidase activity)

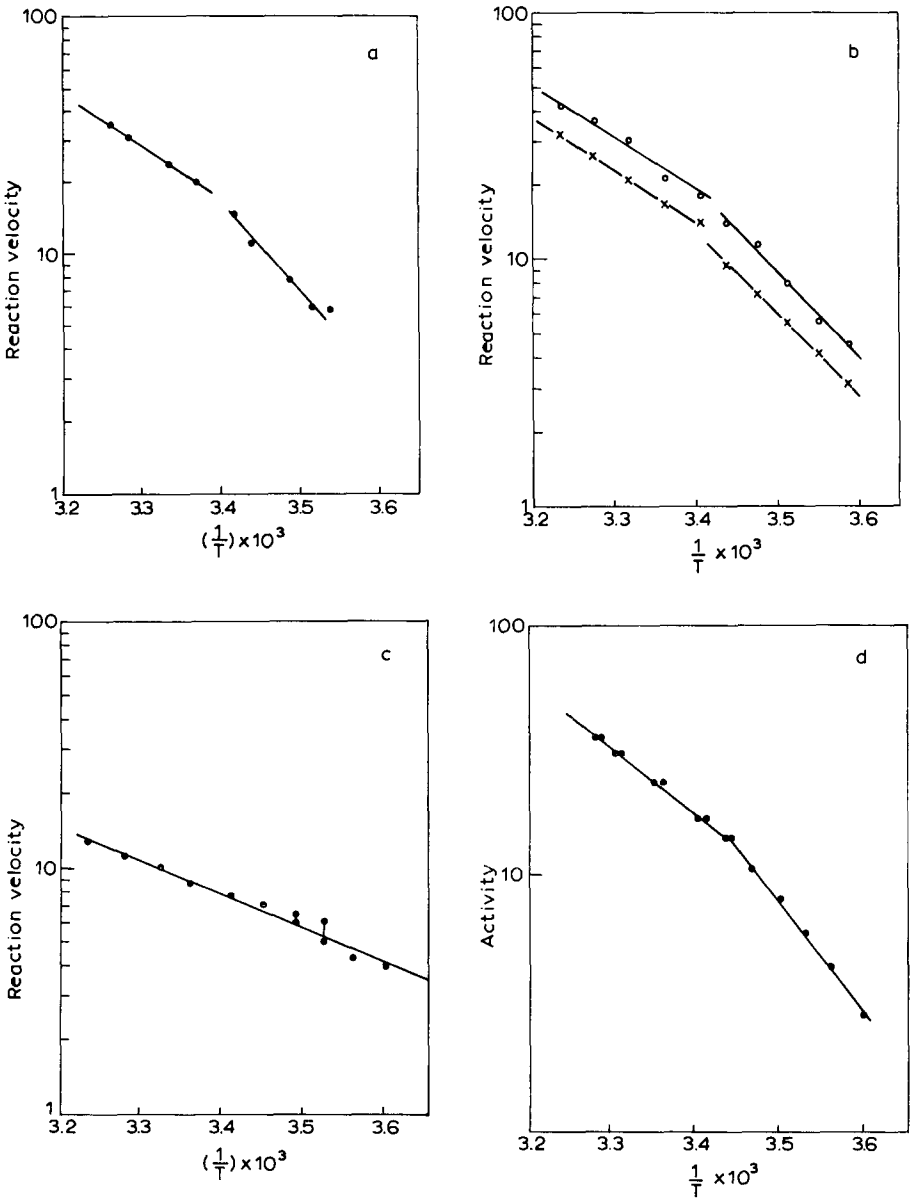

Fig. 7. Arrhenius plots of dihydro-orotate oxidase activity. a. Particulate preparation of *E. coli*. b. Particulate preparation of *S. typhimurium* grown at 32 °C on glucose minimal salts. Measurements made (×—×) without Triton X-100; (○—○) with 0.1% Triton X-100. c. Phospholipase-treated enzyme of *E. coli*. Preparation was solubilized at pH 8.4 and fractionated with ammonium sulfate (precipitating between 60 and 72% saturation). d. Phospholipase-treated enzyme measured in the presence of Triton X-100. Enzyme was solubilized, fractionated with ammonium sulfate, and filtered on an agarose column.

TABLE V

THE EFFECT OF TRYPSIN ON DIHYDRO-OROTATE OXIDASE ACTIVITY IN BROKEN-CELL PREPARATION IN THE PRESENCE AND ABSENCE OF OROTIC ACID

Conditions: extract made from stationary-phase cells in 40 mM, Tris–HCl, pH 7.6. The ratio trypsin: extract protein is 1:560. Proteolysis was stopped by removing samples directly to assay cuvettes containing trypsin inhibitor. The 0 min samples received a mixture of trypsin and trypsin inhibitor.

Activity	Orotate mM during proteolysis	Relative activity					
		Time (min):					
		0	2	4	6	8	10
Oxidase	+	100	58	40	25	16	12
Oxidase	–	100	44	44	26	14	10
DCIP reduction	+	100	62	45	29	22	—

in cell-free extracts with that in whole cells treated so as to make the outer surface of the cytoplasmic membrane accessible. It was assumed that in the extracts, the inner surface of the cytoplasmic membrane would be accessible. The two activities of dihydro-orotate oxidase are equally sensitive to trypsin (Table V). Their half-life is 2–4 min at a trypsin–protein ratio about one fifth of that used by others [26]. Since there is no evidence that the DCIP-reduction requires a protein intermediate this suggests that the dihydro-orotate oxidase itself is vulnerable to trypsin. Orotate, the product of dihydro-orotate oxidase catalysis does not protect the enzyme against trypsin inactivation as it does against thermal inactivation.

To test the accessibility of dihydro-orotate oxidase from the external side of the cytoplasmic membrane, the outer layers of the cell envelope of *E. coli* were modified by two current methods: the Tris–EDTA method of Leive and Kollin [28] and the cold osmotic shock treatment of Neu and Heppel [29]. For this experiment, we

TABLE VI

EFFECT OF TRYPSIN ON DIHYDRO-OROTATE OXIDASE ACTIVITY IN WHOLE CELLS

Effect of trypsin on dihydro-orotate oxidase activity in whole cells grown at 32 °C on minimal salts medium containing 8 μg uracil. One third of the cells was washed at room temperature with 10 mM phosphate buffer, pH 8.0; one third was washed according to Leive and Kollin [28]; one third was cold osmotic shocked according to Neu and Heppel [29]. The cell density was the same in all, i.e., ten times that of the culture at harvest. Two 1 ml samples of each suspension were taken, 100 μg trypsin was added in 0.1 ml to one sample, and then 0.02 ml $CaCl_2$ 500 mM to all. After 2 h at 37 °C, 0.1 ml of trypsin inhibitor 1 mg/ml was added to the trypsinized cells and 0.2 ml of pre-mixed trypsin–trypsin inhibitor to the control. Assays were done on 0.05 ml aliquots in reaction mixtures containing 1 mM EDTA.

Activity	Phosphate-washed cells		Tris–EDTA treated cells		Cold osmotic shocked cells	
Trypsin:	–	+	–	+	–	+
Oxidase	100	92	100	85	100	70
DCIP reduction	100	100	100	75	100	78

used 10 times as much trypsin as for the preceding experiment. Table VI shows that in 2 h of incubation trypsin causes a relatively small decrease in activity which is very likely to be due to lysis of a fraction of the cells [30].

The greater sensitivity of the oxidase and the oxidative system in extracts suggests that the enzyme and the respiratory chain linked with it are on the inner side of the cytoplasmic membrane and do not extend all the way across the thickness of the membrane as indicated in Fig. 8.

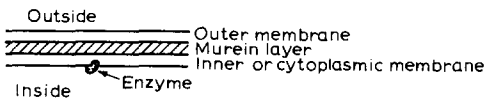

Fig. 8. Suggested location of the oxidase and the respiratory system linked with it.

The enzyme most probably has hydrophobic sites extending into the hydrocarbon layer of the membranes. The bonds formed at these sites probably predominate in stabilizing the association, although ionic bonds may also play a role.

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