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THE PREPARATION AND PROPERTIES OF THE MEMBRANAL DPNH DEHYDROGENASE FROM *ESCHERICHIA COLI*

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SUMMARY

1. The membrane bound DPNH oxidase of *Escherichia coli* can reduce the artificial electron acceptors: ferricyanide, dichlorophenolindophenol (DCIP) and menadione. All three are reduced by the flavoprotein of DPNH oxidase, but at different sites of the enzyme.

2. Freeze-drying of the bacterial membranes causes a selective detachment of DPNH dehydrogenase (DPNH:(acceptor) oxidoreductase, EC 1.6.99.3) from the membranes. This solubilization is accompanied by a decrease of $K_m(K_3Fe(CN)_6)$ from 2.0 to 0.25 mM, while no change is detected in K_m(DPNH). This enzyme is not the DPNH diaphorase found in the bacteria.

3. DPNH dehydrogenase of *E. coli* is a metalloflavoprotein, containing non-heme iron, labile sulfide, FMN and FAD.

4. Reduction of the enzyme with DPNH in the absence of electron acceptor (ferricyanide or DCIP) causes a rapid and irreversible change to a less active state, Form II. Form II is characterized by a higher K_m(DPNH) and slower $v_{max.}$, while the $K_m(K_3Fe(CN)_6)$ remains unchanged.

5. The transformation of the enzyme to Form II is accompanied by the reduction of the non-heme iron component. The role of non-heme iron in the enzymic reaction is discussed.

INTRODUCTION

Following a suggestion of LION AND AVI-DOR[1], it was shown by GUTMAN, SCHEJTER AND AVI-DOR[2] that oxygen has a deletorious effect on the DPNH oxidase activity of dried membranes from *Escherichia coli*. Though this inactivation cannot be correlated with the loss of viability detected in whole bacteria under the same conditions[3,4], it provides a tool for investigating the properties of this enzymic system.

It was already suggested[2] that the flavoprotein region of DPNH oxidase is the oxygen sensitive component of the oxidase system. In this work we describe the

Abbreviations: DCIP, dichlorophenolindophenol; PMS, phenazine methosulfate; NQNO, 2-*N*-nonylhydroxyquinoline-*N*-oxide.

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properties of the membrane bound DPNH dehydrogenase (DPNH: (acceptor) oxidoreductase, EC 1.6.99.3) of *E. coli*, and present a method for solubilization of the enzyme. The composition and properties of the enzyme are described.

EXPERIMENTAL PROCEDURE

Chemicals. All chemicals, of highest purity available, were obtained commercially.

Instrumentation. Absorbances were measured in Cary model-15 or Beckman-BD spectrophotometers. Respiration was measured with a Clark oxygen electrode. Lyophilization was carried out at a pressure of 20–40 mTorr.

Enzymic estimations. All measurements were done at room temperature. The reaction mixtures contained Tris–HCl buffer, 0.05 M (pH 7.4), substrate and electron acceptor at the desired concentrations. DPNH: 5–200 μM; $K_3Fe(CN)_6$: 100–1000 μM; menadione: 30–50 μM; dichlorophenolindophenol (DCIP): 40 μM. The reaction was started by the addition of the enzyme. The reduction of menadione and DCIP was measured in the presence of 3 mM KCN.

Enzymic activities are expressed in nequiv of donor oxidized in 1 min per mg of protein.

DPNH oxidase activity was measured spectrophotometrically at 340 mμ, or by determining oxygen consumption with an oxygen electrode.

DPNH–menadione reductase was measured at 340 mμ, or with the oxygen electrode, because the menadiol produced by the enzymic reduction was found to undergo very rapid autoxidation.

DPNH–$K_3Fe(CN)_6$ reductase was measured spectrophotometrically at 420 mμ.

DPNH–DCIP reductase was measured spectrophotometrically at 660 mμ.

Succinate oxidase, D- and L-lactate oxidase activities were measured with the oxygen electrode.

Succinate dehydrogenase and lactate dehydrogenase were measured by recording the rate of oxygen disappearance after inhibition of respiration by 3 mM KCN, and addition of phenazine methosulfate (PMS)[5] in excess.

Chemical determinations. Non-heme iron was estimated according to MASSEY[6]; labile sulfide as given by BRUMBY, MILLER AND MASSEY[7]; and FMN and FAD with the technique of BRUCH[8].

Total protein was measured by the method of GOA[9].

Preparation of the enzyme

Bacteria. E. coli E/65 was a kind gift of Dr. LION from the Israel Institute for Biological Research, Nes Ziona. The bacteria were grown in broth containing: Bactopeptone, 1%; yeast extract, 0.5%; glucose, 0.2%; and K_2HPO_4, 0.2%. Growth conditions were: 37° at an aeration rate of 0.3 μmole O_2/min per l. The bacteria were harvested after 16 h, washed twice with tap water and then twice with bidistilled water. The bacteria could be kept as a frozen mass at —20° for several months without any damage to their enzymic activities.

Preparation of membranes

The bacteria were sonically disintegrated. The temperature during sonication was kept below 4°. Debris were sedimented at 50000 $\times$ *g* during 20 min and light

membranes were sedimented at 150000 × *g* during 90 min. The light membrane pellet was resuspended in water and sedimented under the same conditions.

Lyophilization. Light membrane suspensions at concentrations of 30 mg protein/ml were distributed in 1–2-ml portions in neutral glass ampules. The material was shell-frozen at −60° and rapidly attached to the lyophilizer. After completion of drying, the ampules were sealed under vacuum and stored at −60°.

DPNH dehydrogenase was prepared from lyophilized light membranes. The dried membranes were resuspended in bidistilled water for a few minutes and centrifuged at 150000 × *g* during 90 min. The supernatant is defined as DPNH dehydrogenase.

RESULTS

Properties of the DPNH oxidase system

The DPNH oxidase system is located in the membranal fraction of the bacteria. This system can reduce oxygen, menadione, DCIP and ferricyanide with DPNH as an electron donor. The specific activities with respect to the various electron acceptors are: respiration, 500 nequiv/min per mg; ferricyanide reduction 2000 nequiv/min per mg; DCIP reduction 350 nequiv/min per mg and menadione reduction 650 nequiv/min per mg.

The effect of KCN, dicumarol, 2-*N*-nonylhydroxyquinoline-*N*-oxide (NQNO) and atebrin on enzymic activities of the membranal enzymes were tested. KCN inhibited respiration at 2–3 mM, had no effect on ferricyanide reduction, and stimulated DCIP reduction by 30–40 %. The reduction of menadione was not affected by cyanide, and menadione restored respiration in cyanide treated membranes.

Both dicumarol and NQNO inhibited competitively menadione reduction with K_i values of 90 μM and 0.1 μM respectively; atebrine inhibited menadione reduction non-competitively, with $K_i = 370$ μM. These three compounds inhibited respiration to 50 % at a concentration equal to K_i for menadione reduction.

Contrary to menadione reduction, the reaction with ferricyanide was insensitive to dicumarol and NQNO. The insensitivity to these two inhibitors could arise from a much higher affinity of ferricyanide than dicumarol or NQNO for a common site. However, this is in disagreement with the observed K_m values, *viz*: $K_m(K_3Fe(CN)_6) = 2$ mM and K_i(dicumarol) = 90 μM. Thus, it appears that the site of ferricyanide reduction lies between the substrate site and the site of dicumarol and NQNO inhibitory effect.

It is well known that quinones are reduced by flavoproteins; therefore, we conclude that ferricyanide is also reduced by the flavoprotein, but at a site which differs from the quinone reducing site.

To clarify whether other carriers in the bacterial electron transport system can reduce ferricyanide, the electron flow from the flavoprotein to oxygen was abolished by ultraviolet illumination[10]. If ferricyanide reduction occurred at a membranal site different from that of the flavoprotein, ultraviolet illumination should abolish some of the ferricyanide reduction. As shown in Fig. 1, this was not the case.

The effect of dicumarol on DCIP reduction was biphasic (Fig. 2). The effect of NQNO on DCIP reduction was similar to that of dicumarol.

The effect of freezing and drying on the electron transport system of E. coli

In the investigation of the effect of freezing and drying, two membranal activitites were studied side by side: DPNH oxidase and succinate oxidase. Each of these two systems has a specific flavoprotein which is oxidized by the same cytochrome chain.

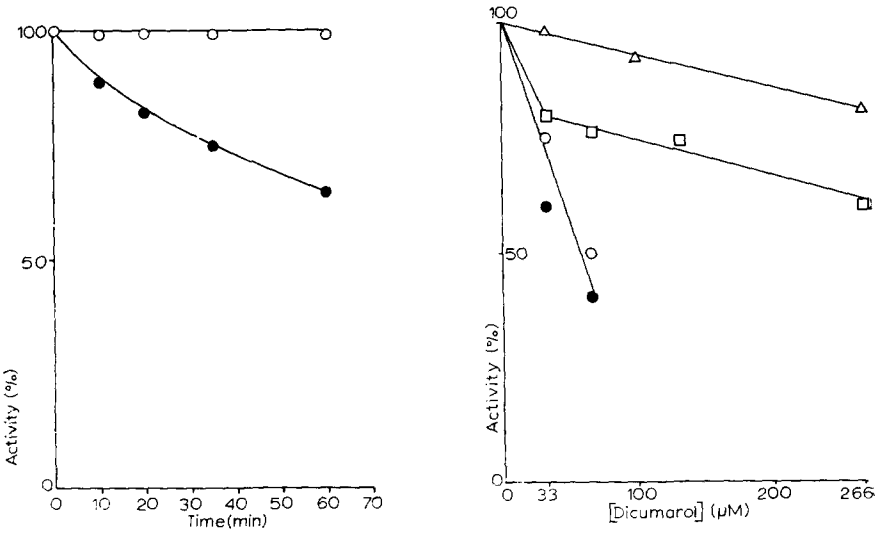


Fig. 1. The effect of ultraviolet illumination on the DPNH oxidase and DPNH–$K_3Fe(CN)_6$ reductase activities of *E. coli* membranes. The illumination was carried out from 3 cm at 0° with a 9-W, ultraviolet lamp with maximal output at 336 mμ. Activities measured as given under EXPERIMENTAL PROCEDURE. ○—○, DPNH–$K_3Fe(CN)_6$ reductase; ●—●, DPNH oxidase.

Fig. 2. The effect of dicumarol on DPNH oxidation by *E. coli* membranes with different electron acceptors. Activities measured as described under EXPERIMENTAL PROCEDURE. The electron acceptors used were: △—△, $K_3Fe(CN)_6$; □—□, DCIP; ○—○, menadione; ●, oxygen.

TABLE I

THE EFFECT OF FREEZING AND FREEZE-DRYING ON OXIDASE ACTIVITIES OF *E. coli* MEMBRANES

In Expt. 4 whole bacteria were treated and the enzymic activity was determined on the sonicate of the bacteria. Activities were determined as described in EXPERIMENTAL PROCEDURE.

Expt. No.	*Activity*	*Suspending medium*	*Spec. act. (nequiv/min per mg)*		
			Membranes	*Frozen membranes*	*Dried membranes*
1	Succinate oxidase	Water	315	275	310
	Succinate dehydrogenase	Water	435	385	375
2	DPNH oxidase	Water	920	450	315
	DPNH dehydrogenase	Water	2050	2100	1850
3	DPNH oxidase	Sucrose, 0.25 M	1100	800	—
	DPNH dehydrogenase	Sucrose, 0.25 M	1500	1800	—
4	DPNH oxidase	Whole bacteria	410	410	180
	DPNH dehydrogenase	Whole bacteria	1800	1750	1050

We investigated the effect of freezing and drying on the flavoprotein activity and on the integrity of the electron transport system which was measured by the oxidase activity. The results are summarized in Table I.

DPNH oxidase is affected both by freezing and by drying. It should be noted that the effect of freezing is less marked when the membranes are frozen from 0.25 M sucrose and completely undamaged when whole bacteria are frozen. On the other hand drying affects the DPNH oxidase of whole bacteria and of aqueous suspensions of membranes.

The decrease of DPNH-oxidase activity is parallelled by variation of K_m-($K_3Fe(CN)_6$) of the membranes. For freshly prepared or frozen membranes, 2.0 and 1.9 mM respectively, while for dried membranes $K_m = 0.5$ mM. These changes caused by dehydration indicate an irreversible change in the region of the flavoprotein ferricyanide reducing site. When freeze-dried membrane suspensions were centrifuged at 150000 $\times$ g during 150 min, a clear yellow supernatant was obtained, with DPNH–$K_3Fe(CN)_6$-reductase activity ($K_m(K_3Fe(CN)_6) = 250 \pm 50$ μM). This fraction did not show the following activities, that were present in the intact membrane: DPNH oxidase, succinate oxidase, succinate dehydrogenase, lactate oxidase, lactate dehydrogenase, malate oxidase, malate dehydrogenase. Because of the low yield, less than 100 mg protein for 100 g of packed bacteria, no further purification steps were undertaken. This fraction is defined as *E. coli* DPNH dehydrogenase. The distribution of activities during the extraction procedure is presented in Table II.

TABLE II

THE DISTRIBUTION OF ENZYMIC ACTIVITIES DURING THE PREPARATION OF DPNH DEHYDROGENASE FROM DRIED *E. coli* MEMBRANES

	mg protein	*DPNH oxidase*		*DPNH–$K_3Fe(CN)_6$ reductase*	
		Spec. act.	*Total act.*	*Spec. act.*	*Total act.*
Dried membranes	930	500	$4.6 \cdot 10^5$	1400	$13 \cdot 10^5$
Supernatant 150000 $\times$ g during 90 min	94	0	0	4600	$4.6 \cdot 10^5$
Precipitate 150000 $\times$ g during 90 min	840	510	$4.3 \cdot 10^5$	1040	$8.4 \cdot 10^5$

It may be noticed that the DPNH oxidase activity was unaffected by removal of the solubilized DPNH dehydrogenase. This indicates that the enzyme which was detached during drying does not function any more as an electron donor to the cytochrome chain. It should be emphasized that no enzyme was released from membranes that had not undergone previous drying. What more, digestion with phospholipase A or sonication with digitonin failed to release the enzyme from the membranes. The amount of DPNH dehydrogenase which can be extracted from the dried membranes varies from 30 to 50 %, of the dried membranes activity.

The origin of DPNH dehydrogenase

The sonicated bacteria demonstrated very high activity of DPNH–$K_3Fe(CN)_6$ reductase: 11000 nequiv/min per mg. Most of this activity remained in solution after

sedimenting the membranes. The soluble enzyme oxidized DPNH with DCIP, menadione and $K_3Fe(CN)_6$ as acceptors. Inhibition by atebrin suggested that this activity is carried out by a flavoenzyme. Because of these characteristics we shall refer to the enzyme as DPNH diaphorase.

The K_m(DPNH) values of the membrane bound enzyme are very low, K_m = 12 μM, with either oxygen or ferricyanide as acceptors. K_m(DPNH) for DPNH dehydrogenase is of the same magnitude, 8 to 12 μM, while that of DPNH diaphorase is 320 μM. These results permit to place DPNH dehydrogenase in the same class with the membranal activities. Furthermore, the affinity of DPNH diaphorase for $K_3Fe(CN)_6$ is very high (K_m = 15 μM) while that of DPNH dehydrogenase is much lower (K_m = 250 ± 50 μM).

A third distinction between DPNH diaphorase and the membranal enzyme is evidenced by dicumarol inhibition (Fig. 3). It can be seen that membranal DPNH–$K_3Fe(CN)_6$ reductase and DPNH dehydrogenase are both nearly unaffected by dicumarol, while DPNH diaphorase is distinctly sensitive.

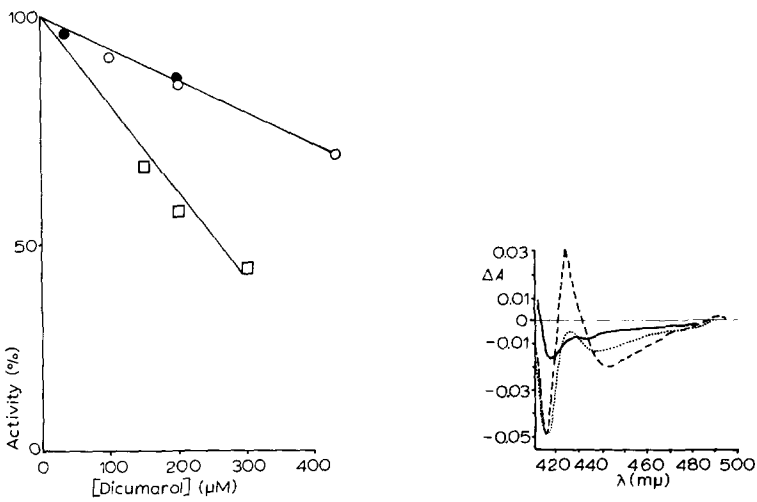


Fig. 3. The effect of dicumarol on DPNH oxidation by DPNH dehydrogenase, membrane bound DPNH–$K_3Fe(CN)_6$ reductase, and DPNH diaphorase from *E. coli*. O—O, membrane bound DPNH–$K_3Fe(CN)_6$ reductase; ●—●, solubilized DPNH dehydrogenase; □—□, DPNH diaphorase.

Fig. 4. The difference spectrum of DPNH dehydrogenase. The enzyme, 0.6 mg/ml in 0.05 M Tris (pH 7.4) was reduced with DPNH and the spectrum of the reduced enzyme was measured against that of the oxidized enzyme. ———, 3 min of reduction; ·····, 30 min of reduction; – – – –, reduced with dithionite.

Spectral properties and constituents of DPNH dehydrogenase

The difference spectrum of oxidized *vs.* reduced DPNH dehydrogenase is shown in Fig. 4. Reduction by DPNH causes the appearance of two troughs at 410–415 mμ and 440 mμ, which are assigned to non-heme iron, and flavin, respectively. The peak at 428 mμ is attributed to minor concentration of cytochrome *b* which is reduced only with dithionite.

The spectra of oxidized DPNH dehydrogenase measured at increasing pH values, showed a decrease of absorbance at 410 mμ, characteristic of non-heme iron[11].

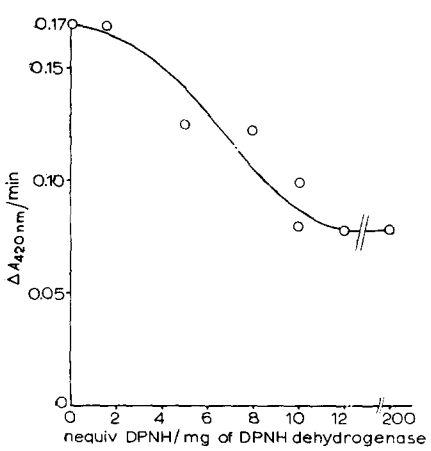
DPNH dehydrogenase was found to contain non-heme iron, flavin and labile sulfide. Analysis of four different batches gave the following results: Non-heme iron: 14–16 natom/mg protein; labile sulfide: 11–13 natom/mg; FMN: 0.3 nmole/mg; and FAD: 0.2 nmole/mg.

The effect of reduction on the catalytic properties of DPNH dehydrogenase

The rate of the enzymic reaction depended on the sequence of addition of DPNH and electron acceptors to the enzyme. When electron acceptors, $K_3Fe(CN)_6$ or DCIP, were added before DPNH, the rate was twice as high as that measured when DPNH was added prior to the electron acceptor. Increasing the length of the pretreatment period with DPNH, from 0.5 to 10 min, had no effect on the extent of the inhibition. DPNH from different sources gave the same results. DPN^+ had no effect on the enzymic activity.

DPNH dehydrogenase was treated with 10 μM DPNH for 2 min, and $K_3Fe(CN)_6$ was added in great excess (700 μM). After all the DPNH was oxidized, the enzyme was kept with the remaining $K_3Fe(CN)_6$ for 5 to 25 min, substrate amounts of DPNH were added and the rate of the reduction was measured. No reversal of inhibition was detected. These results clearly distinguish this phenomenon from inhibition by excess of substrate.

The irreversibility of the process makes it possible to estimate the amount of DPNH participating in this process. DPNH dehydrogenase was incubated for 2 min with a very small amount of DPNH. After this, 700 μM $K_3Fe(CN)_6$ were added, followed by substrate amounts of DPNH, and the rate of the reaction was measured. As seen in Fig. 5 about 10 nequiv of DPNH per mg protein caused maximal inhibition; this is nearly equivalent to the non-heme iron present in the enzyme. For convenience,



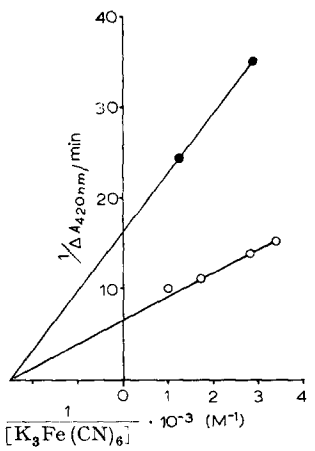


Fig. 5. Titration of the amount of DPNH needed to transform DPNH dehydrogenase to its Form II. DPNH dehydrogenase, 0.03 mg/ml in 0.05 M Tris (pH 7.4) was treated with increasing concentrations of DPNH, from 0.1 to 140 μM, for 2 min; after this, $K_3Fe(CN)_6$ was added to final concentration of 700 μM. After another 2 min DPNH (150 μM) was added and the rate of the reaction was measured. Note the discontinuity of the abscissa and the titration curve.

Fig. 6. Determination of $K_m(K_3Fe(CN)_6)$ for DPNH dehydrogenase and DPNH dehydrogenase in Form II. The transformation to Form II was carried out as described in the text. O—O, DPNH dehydrogenase; ●—●, DPNH dehydrogenase (II).

DPNH dehydrogenase pretreated with DPNH will be referred as DPNH dehydrogenase (II).

The nature of the inhibition of DPNH dehydrogenase (II) activity

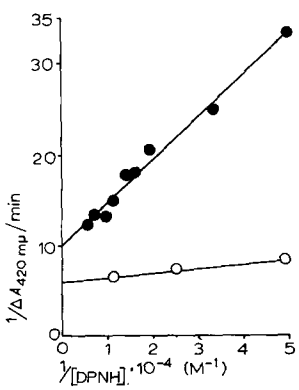
The nature of the inactivation was investigated by measuring K_m(DPNH) and $K_m(K_3Fe(CN)_6)$ for DPNH dehydrogenase and for DPNH dehydrogenase (II). It is seen from Fig. 6 that the transformation to Form II does not affect $K_m(K_3Fe(CN)_6)$, indicating that no change occurred in the reducing site. The oxidizing site of DPNH dehydrogenase is the one which reflects the transformation; this is shown in Fig. 7.

The role of iron in enzymic activity

The similarity between the amounts of DPNH causing full transformation to Form II and the non-heme iron content of the latter, lead to the assumption that in Form II the non-heme iron of the enzymes is in the ferrous state; thus, chemical reduction of DPNH dehydrogenase should lead to formation of DPNH dehydrogenase (II). Ascorbate does not reduce DPNH dehydrogenase, but in the presence of *o*-phenanthroline, reduction of the non-heme iron was detected by the formation of the red colour of Fe^{2+}–*o*-phenanthroline complex.

The rate of the red colour appearance was slow and full colour intensity was developed only after 30 min. The same red colour appeared when the enzyme was reduced with DPNH in presence of *o*-phenanthroline. The spectrum of the red product is very similar to that of Fe^{2+}–(*o*-phenanthroline)$_3$, with a red shift of 5 mμ (Fig. 8).

The red product was found to be non-dialyzable. This, and the red shift in the spectrum, suggest that the Fe^{2+}–*o*-phenanthroline complex is attached to the enzyme. By assigning the same molar extinction to the enzyme bound complex and Fe^{2+}–(*o*-phenanthroline)$_3$, it was found that only 4–6 natom/mg of non-heme iron reacted with *o*-phenanthroline. Similar results were reported for dihydroorotic dehydrogenase[12].



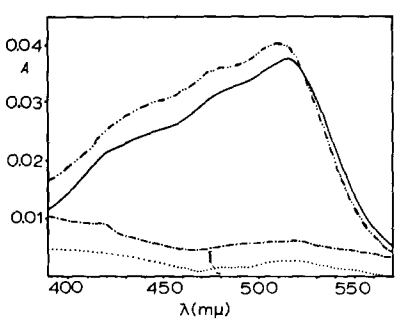


Fig. 7. Determination of K_m (DPNH) for DPNH dehydrogenase and DPNH dehydrogenase (II). The transformation to Form II was carried out as described in the text. O—O, DPNH dehydrogenase (K_m = 8 μM). ●—●, DPNH dehydrogenase (II) (K_m = 43 μM).

Fig. 8. The absorption spectrum of the red color formed by addition of *o*-phenanthroline to the reduced DPNH dehydrogenase. ———, DPNH dehydrogenase, 0.8 mg/ml dialyzed (16 h) against *o*-phenanthroline, 0.5 mM and DPNH, 2 mM; —·—, DPNH dehydrogenase 0.76 mg/ml dialyzed (16 h) against DPNH, 2 mM. —··—, the absorption spectrum of 3.45 μM Fe^{2+}–(*o*-phenanthroline)$_3$; ·····, base line.

The same value was measured when either DPNH or ascorbate were used for reduction. Furthermore, it should be noted that although DPNH causes a rapid transformation to Form II, the rate of Fe^{2+}–*o*-phenanthroline appearance is the same when ascorbate is used. Since binding of Fe^{2+} by *o*-phenanthroline is very fast, we conclude that the slow rate of appearance of the red colour is due to the rate limiting access of *o*-phenanthroline to the non-heme iron site.

The reduction of DPNH dehydrogenase with ascorbate and *o*-phenanthroline caused slow transformation of enzymic activities. The transformation was terminated after 30 min. In the ascorbate–*o*-phenanthroline treated enzyme a higher K_m(DPNH) was measured: 20 instead of 10 μM. This increase of K_m was accompanied by a higher $v_{max.}$ of the reaction. Neither *o*-phenanthroline nor ascorbate alone had effect on K_m(DPNH), or on the rate of enzymic activity. After establishing that reduction of non-heme iron increased K_m(DPNH) similarly to transformation to Form II, we looked for the possibility of transformation to Form II in DPNH oxidase. This was done by treating DPNH oxidase with DPNH in the absence of oxygen. After a few minutes air was let in (or $K_3Fe(CN)_6$ was added) and K_m(DPNH) and the reaction velocities were measured; no increase of K_m(DPNH) was detected, while $v_{max.}$ was found to decrease to 80 % of the original value. However, when *o*-phenanthroline was present during the anaerobic DPNH treatment, K_m(DPNH) was significantly increased from 8 to 43 μM (Fig. 9).

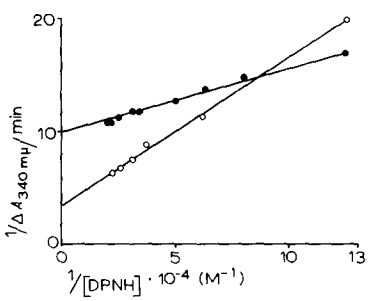


Fig. 9. Determination of K_m(DPNH) for membranal DPNH oxidase after anaerobic treatment of the membranes with *o*-phenanthroline and DPNH. The membranes in 0.05 M Tris (pH 7.4) were flushed three times with N_2 in a Thunberg cuvette and DPNH was added from the side arm. After 3 min air was let in and DPNH oxidation was measured at 340 mμ. O—O, membrane treated with 160 μM *o*-phenanthroline (K_m = 43 μM). ●—●, membrane treated in the absence of *o*-phenanthroline (K_m = 8 μM).

DISCUSSION

The electron acceptors of the flavoprotein of DPNH oxidase

From the three electron acceptors which are reduced by the membrane bound DPNH dehydrogenase: DCIP, menadione and ferricyanide, the latter is the best suited to characterize the flavoprotein. This is based on the following facts: (1) Menadione, the reducible moiety of bacterial naphtoquinones[13], is reduced at the same site as the membranal quinones. This is suggested from the competitive inhibition of its reduction by dicumarol and NQNO. NQNO is known to inhibit cytochrome *b* reduction in *E. coli*[14]. These two inhibitors have the same K_i for both respiration and menadione reduction. The similar rates of menadione reduction and respiration are

much slower than the reduction of ferricyanide. This slow rate may reflect a rate limiting oxidation of reduced flavoprotein by membranal quinones. (2) The effect of inhibitors on DCIP reduction is bi-phasic (Fig. 2). The first phase is due to non-enzymic reduction of DCIP by the membranal quinoles, while the second phase represents the direct reduction by the enzyme on a site with properties similar to the ferricyanide reducing site.

The high rate of ferricyanide reduction represents the highest capacity of the enzyme to conduct electrons from substrate to the acceptor. This ability to reduce ferricyanide is regarded as an indication of native condition of other DPNH dehydrogenases[15].

Preparation of DPNH dehydrogenase

The common methods for releasing enzymes from particles are those which affect the lipid fraction of the particles, such as treatment with detergents[16] or phospholipase A (ref. 17). In our experiments, neither phospholipase A nor digitonin were able to release DPNH dehydrogenase from the bacterial membrane. On the other hand, the dehydration of the membranes was found suitable for this purpose. With this procedure, up to 50 % of the membrane-bound activity can be released, and the soluble preparation obtained, is free from other membrane-bound activities such as succinate dehydrogenase, malate dehydrogenase and D- and L-lactate dehydrogenases. The yield of the enzyme based on the activity is reproducible, but the amount of protein solubilized by this procedure, varies among preparations. The nature of other non-enzymic proteins which are released together with the DPNH dehydrogenase, is unknown.

An important feature of the process leading to solubilization of DPNH dehydrogenase, is that $K_m(K_3Fe(CN)_6)$ of the flavoenzyme decreases during its release. This can be taken as an indication of unmasking of the $K_3Fe(CN)_6$-reducing site as the enzyme is detached from the membranal structure. The DPNH-oxidizing site is unaffected by this process.

Properties of DPNH dehydrogenase

DPNH dehydrogenase of *E. coli* is a metalloflavoprotein. Its spectrum, and the effect of pH on it are very similar to those of mitochondrial DPNH dehydrogenase. *E. coli* DPNH dehydrogenase contains FMN, FAD, non-heme iron and labile sulfide, which are common to well recognized metalloflavoenzymes, like mammalian DPNH dehydrogenase[18] or yeast DPNH dehydrogenase[19].

A membrane bound DPNH dehydrogenase from *E. coli* was solubilized with deoxycholate[16]. Similarly to our preparation, this enzyme is insensitive to *p*-chloromercuribenzoate, but it is inhibited by *o*-phenanthroline and has a much higher K_m-(DPNH) value[20].

The incomplete reaction of non-heme iron with *o*-phenanthroline can be caused either by incomplete reduction of all non-heme iron atoms, or by the fact that some non-heme iron atoms are unaccessible to *o*-phenanthroline. The fact that the same amount of non-heme iron reacts when DPNH or ascorbate are used, indicates that the reaction with *o*-phenanthroline may be the limiting factor. Further information is derived from the fact that the rates of complex formation are similar when DPNH or ascorbate are used for reduction.

Thus we conclude that the slow rate of complex formation is due to the slow rate of access of *o*-phenanthroline to the non-heme iron site.

The effect of reduction on enzymic activity

The transformation to DPNH dehydrogenase (II) is accompanied by the reduction of the enzyme. This conclusion is based on the following results: (1) No transformation occurs when electron acceptors are present while treating the enzyme with DPNH. (2) The transformation is caused by very low DPNH concentrations, and the amounts of DPNH needed are of the same order of magnitude as the non-heme iron content of the enzyme. (3) The transformation is very rapid. ROSSI *et al.*[21] reported that mitochondrial DPNH dehydrogenase is fragmented in the presence of DPNH to smaller units, but the process described by these authors is much slower than transformations to Form II. (4) The characteristic increase of K_m(DPNH) is detected when DPNH dehydrogenase of DPNH oxidase are reduced in the presence of *o*-phenanthroline that prevents reoxidation of the ferrous iron. (5) The transformation to Form II is not an inhibition by excess of DPNH, since no reconversion from Form II can be detected when all the DPNH in the solution is oxidized.

DPNH dehydrogenase (II) differs from the native enzyme in two main aspects: it has a markedly lower affinity for DPNH, and a much lower $v_{max.}$. These two facts can be interpreted by attributing to the non-heme iron two functions: one, to bind DPNH, and the other, to act as an intramolecular electron transport system.

Binding of DPNH to aqueous solutions of ferric iron has been shown to occur[22]. In this case, a complex is formed, and it decomposes into ferrous ions and DPN^+. Since ferrous iron does not appear to form a complex with DPNH, reduction of non-heme iron in the enzyme will decrease its affinity to DPNH. We may regard the slower rate of oxidation catalyzed by DPNH dehydrogenase (II) as a reaction mediated only by the flavins, such as in the processes described by FOX AND TOLLIN[23]. When DPNH dehydrogenase is brought to Form II in the presence of *o*-phenanthroline, we still detect an increase of K_m(DPNH) but the velocity of the reaction is faster. This, too, can be explained by the fact that the ferric–*o*-phenanthroline complexes are reduced by DPNH at a rate faster than the reduction of $Fe^{3+}(H_2O)_6$ by DPNH (ref. 24). The participation of non-heme iron in the binding of DPNH is suggested by the fact that dipyridyl inhibits competitively *E. coli* DPNH–menadione reductase[20].

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