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Studies on the succinate dehydrogenating system. Interaction of the mitochondrial succinate-ubiquinone reductase with pyridoxal phosphate

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The inhibitory effect of pyridoxal phosphate on the Triton X-100 solubilized purified bovine heart succinate-ubiquinone reductase (Choudhry, Z.M., Gavrikova, E.V., Kotlyar, A.B., Tushurashvili, P.R. and Vinogradov, A.D. (1985) FEBS Lett. 182, 171–175) was studied. The kinetics of the enzyme inactivation by pyridoxal phosphate was found to be strongly dependent both qualitatively and quantitatively on the concentration of the protein-detergent complexes. In the diluted system the inactivation of the ubiquinone-depleted enzyme was completely prevented by the saturating concentrations of Q_2, carboxin, thenoiltrifluoroacetone and pentachlorophenol, i.e., by the substrate and specific inhibitors of the enzyme. The protective effects of Q_2 and the inhibitors was employed to quantitate the affinities of the ligands to their specific binding sites. Strong difference in the affinity of Q_2 to the reduced and oxidized enzyme was found. When the soluble reconstitutively active succinate dehydrogenase was treated with pyridoxal phosphate, the reactivity of the enzyme towards low ferricyanide concentrations and its reconstitutive activity was significantly protected against aerobic inactivation.

Introduction

Several models for the functioning of ubiquinone in the mammalian respiratory chain have been suggested [1–8]. Until recently the kinetic studies of ubiquinone reduction by the respiratory chain-linked dehydrogenases have been hampered by the presence of significant amount of functionally active bound ubiquinone in the resolved preparations of succinate- [9,10] and NAD · H-ubiquinone [11] reductases (complex II and I, respectively). The quantitative data on the affinities of the substrate and product to the enzyme seems to be a prerequisite for any appropriate model of the reaction mechanism on the acceptor sites of the respiratory chain-linked dehydrogenases.

Recently a new method for the preparation of the soluble highly purified succinate-ubiquinone reductase from bovine heart submitochondrial particles has been developed in this laboratory [12]. An important feature of the preparation is that it does not catalyze the reduction of some artificial electron acceptors (DCIP, Wurster's blue) by succinate in the absence of added ubiquinone

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Abbreviations: carboxin, 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide; PMS, *N*-methylphenazonium sulphate; DCIP, 2,6-dichlorophenolindophenol; Wurster's blue, a semiquinone diimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Q_n, homologs of ubiquinone having *n* isoprenoid units in position 6 of the quinone ring; SDS, sodium dodecyl sulfate.

or its lower homologs, thus indicating that the preparation is essentially free of the functionally active bound ubiquinone. The kinetic studies of ubiquinone reduction by the purified succinate-ubiquinone reductase [12,13] suggest the presence of a specific quinone binding site which is formed by the succinate dehydrogenase [14] (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) and one or two low-molecular-weight peptides [15–20] (operationally named QP_s [15]).

We have recently reported that pyridoxal phosphate inactivates the soluble succinate-ubiquinone reductase causing the dissociation of the succinate dehydrogenase from the complex [21]. An interesting property of such inactivation is that Q_2 protects the enzyme against inhibitory effect of pyridoxal phosphate [21]. It seemed that the protection of succinate-ubiquinone reductase by the substrate or by the specific inhibitors, if any, might be a useful tool for the quantitation of their affinities to the enzyme. This report describes the kinetics of pyridoxal phosphate-induced inhibition of the soluble and particles-bound succinate-ubiquinone reductase. It will be shown that dramatic difference in the quinone binding properties exists between the reduced and oxidized enzyme. In addition the protective effect of pyridoxal phosphate treatment on aerobic inactivation of the reconstitutive activity of the soluble succinate dehydrogenase will also be reported.

Materials and Methods

Succinate-ubiquinone reductase was prepared using a procedure developed in this laboratory [12]. To obtain the protein free of contaminating succinate the following modifications were introduced. The enzyme was eluted from a calcium-phosphate gel by 20 mM potassium malonate/0.1% Triton X-100/0.1 mM EDTA, and after precipitation by ammonium sulfate it was dissolved in the same solution containing 0.1 M potassium malonate. The protein was dialyzed for 1 h at 20°C against 5 mM potassium phosphate (pH 7.8)/0.1 mM EDTA, the buffer was changed and dialysis was continued for 2 h at 0°C. The dialyzed protein was slowly (0.3 ml/min) passed through the column (30 × 1.5 cm) packed with Sephadex G-50 (coarse) and equilibrated with the buffer used for the dialysis. The combined fractions containing no malonate were collected and stored in liquid nitrogen.

The soluble reconstitutively active succinate dehydrogenase [16] and oxaloacetate-free submitochondrial particles [22] were prepared according to the published procedures. The succinate: acceptor reductase activities were measured at 25°C using PMS and DCIP [23], ferricyanide [24,25], Wurster's blue [26] or Q_2 and Wurster's blue [13] as acceptors in a standard assay mixture comprising 20 mM phosphate/20 mM succinate/0.1 mM EDTA (potassium salt, pH 7.8)/0.004% Triton X-100 (for details see legends to the figures and tables). 5 mM sodium azide was added to the assay mixture when the activity of submitochondrial particles was measured to inhibit cytochrome oxidaze. All the activities were expressed as μmol succinate oxidized per min per mg protein. The protein content was determined by the method of Lowry et al. [27] or Gornall et al. [28]. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [29], using 12.5% gels and stained with Coomassie brilliant blue (R-250).

Wurster's blue was prepared from *N,N,N',N'*-tetramethyl-*p*-phenylene diamine [30]. Q_2H_2 was prepared by reduction of Q_2 [31]. The special chemicals were: Triton X-100 from Loba Chemie (Austria), PMS from Lawson (U.K.), DCIP from General Biochemicals (U.S.A.), Q_2 from Ferak (Berlin, G.D.R.) *N*-ethylmaleimide from BDH (U.K.). Carboxin was a kind gift from Prof. H. Lyr (Institute of Plant Protection Research, G.D.R.). All other chemicals used were of highest quality commercially available.

Results

Kinetics of the pyridoxal phosphate-induced inactivation of the soluble succinate-ubiquinone reductase

The time-course of the succinate-ubiquinone reductase inactivation by pyridoxal phosphate is shown in Fig. 1A; the secondary plots demonstrate that the inactivation process can be satisfactorily described by a simple first-order kinetic mechanisms in respect of both the residual enzyme activity and the inhibitor concentration (Fig.

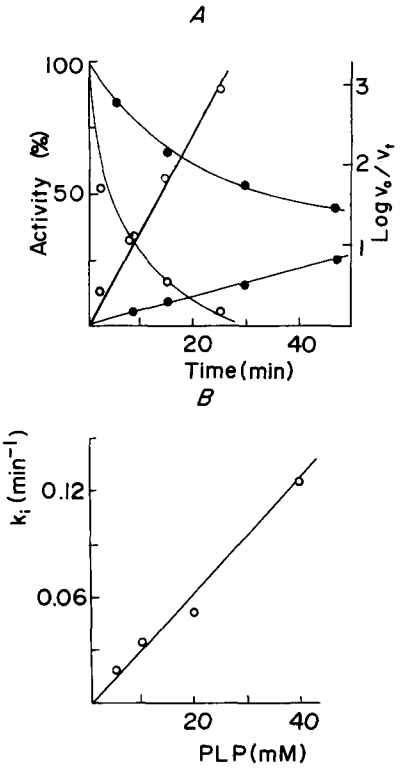

Fig. 1. Kinetics of inhibition of the soluble succinate-ubiquinone reductase by pyridoxal phosphate. (A) The enzyme (10 μg/ml) was incubated at 25°C for the time indicated in a mixture containing 20 mM phosphate, 2 mM malonate, 0.1 mM EDTA (potassium salts, pH 8.0) and 5 (●) or 40 (○) mM pyridoxal phosphate. The proper amounts of the mixture were transfered to the assay cuvettes (2 ml) and after 10 min incubation the carboxin-sensitive succinate-ubiquinone reductase was determined with Q_2 (5 μM) and Wurster's blue (30 μM) as the final electron acceptor. The straight lines are semilogarithmic plots of the activity decay (right ordinate). 100% or the V_0 value corresponds to the original activity of 12. (B) The dependence of the apparent inhibition first-order-rate constant on concentration of pyridoxal phosphate (PLP).

1B). The pyridoxal phosphate-induced inhibition can be reversed by an excess of an aminoacid. When 0.5 M glycine was added to the concentrated pyridoxal phosphate-inhibited enzyme about 80% of the original succinate-ubiquinone reductase was recovered after 10 min of incubation at 25°C. However, when the pyridoxal phosphate-treated enzyme was first diluted 100-times by the addition to the assay system containing glycine, no succinate-ubiquinone reductase activity was recovered. The strong dependence of the reversibility of the inhibition on the protein concentration is actually expected, since the inhibitory effect of pyridoxal phosphate is due to dissociation of succinate dehydrogenase from the complex [21] and apparently no reconstitution of succinate-ubiquinone reductase from the dissociated components occurs in the very diluted system.

We have previously shown that the Triton X-100 solubilized enzyme appears as either carboxin-insensitive succinate-ferricyanide reductase or carboxin-sensitive succinate-ubiquinone reductase depending on the concentration of the protein/detergent complexes in the solutions where the samples for the assay are taken from [12,32]. Since pyridoxal phosphate inhibits the succinate-ubiquinone reductase and not the succinate-ferricyanide reductase [21], it was of interest to find out whether the pseudo-first-order rate constant for pyridoxal phosphate-induced inactivation depends on the protein concentration. Fig. 2 shows that the rate of inactivation by pyridoxal phosphate strongly depends on the protein/detergent concentration. In contrast, when the rate of in-

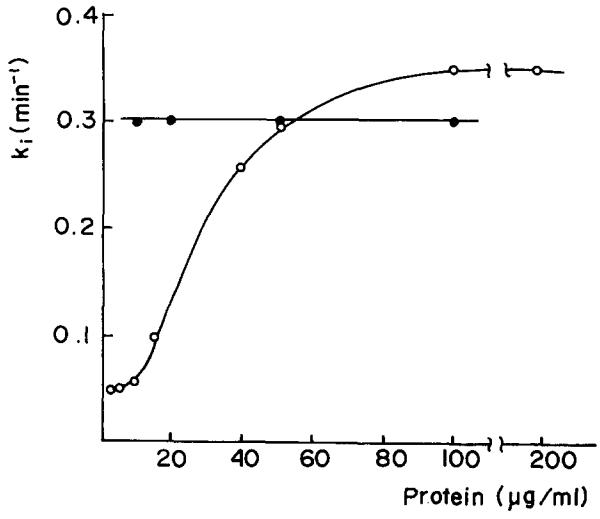

Fig. 2. The dependence of the inhibition first-order-rate constants on the concentration of the soluble succinate-ubiquinone reductase. The enzyme (the protein contents are indicated) was incubated and assayed as described in Fig. 1. (○) 20 mM pyridoxal phosphate was used as the inhibitor; (●) 50 μM *N*-ethylmaleimide was used as the inhibitor (malonate was absent in the incubation medium).

TABLE I

THE PROTECTIVE EFFECT OF Q_2 ON PYRIDOXAL PHOSPHATE-INDUCED INACTIVATION OF THE SUCCINATE-UBIQUINONE REDUCTASE

The enzyme (protein concentration as indicated) was incubated at 25°C in a mixture containing 20 mM phosphate, 20 mM succinate, 0.1 mM EDTA (potassium salts, pH 8.0), pyridoxal phosphate and 50 μM Q_2 (where indicated) for 10 min. The proper amounts of the mixture were then withdrawn to the assay cuvette and the activity was determined after 10 min of incubation. 30 and 10 mM pyridoxal phosphate was added to the preincubation mixture containing 12 and 300 μg of protein/ml, respectively, in order to inhibit the enzyme to approximately the same level (see the difference in pseudo-first-order rate constant at different protein concentrations shown in Fig. 2).

Protein concn. (μg/ml)	Activity remaining (μmol/min per mg)			Inhibition by pyridoxal phosphate (%)	
	no pyridoxal phosphate	plus pyridoxal phosphate		$-Q_2$	$+Q_2$
		$-Q_2$	$+Q_2$		
12	20	9.0	19.6	55	0.4
300	20	6.6	10.0	67	48.0

activation of the same enzyme preparation by *N*-ethylmaleimide was measured the same pseudo-first-order rate constants were determined for any protein concentration (Fig. 2). Since *N*-ethylmaleimide is known to be the dicarboxylate binding site inhibitor of succinate dehydrogenase [33,34], the results shown in Fig. 2 strongly suggest that the conformational state of QP_s or mode of linkage between iron-sulfur-flavoprotein and QP_s depends on an aggregational state of the enzyme/detergent complexes. Indeed, when the protective effect of Q_2 was studied at different protein concentrations it was found that the slow pyridoxal phosphate-induced inhibition (low concentrations of the protein) was almost completely prevented by Q_2, whereas the rapid inactivation which is seen at high protein concentrations was only slightly prevented by Q_2 (Table I).

The protective effect of Q_2 and the specific inhibitors of the pyridoxal phosphate-induced inactivation

The protective effect of Q_2 on pyridoxal phosphate-induced inactivation of the succinate-ubiquinone reductase (Table I) was used to quantitate the binding of quinone and some specific inhibitors to their specific sites on the enzyme. Fig. 3 shows the linear dependence of the pseudo-first-order inactivation rate constant reciprocal on the added Q_2 concentration. The kinetics of the protective effect suggest the presence of a single site for quinone binding. Since an excess of succinate was present and the enzyme rapidly reduce Q_2, the intercept of the straight line with abscissa corresponds to the K_s value for the reversible interaction between Q_2H_2 and the reduced enzyme. Using the same approach as in Fig. 3 (for the details of the kinetic analysis, see Ref. 34), the dissociation constants for the complexes between the enzyme and other ligands were determined (Table II).

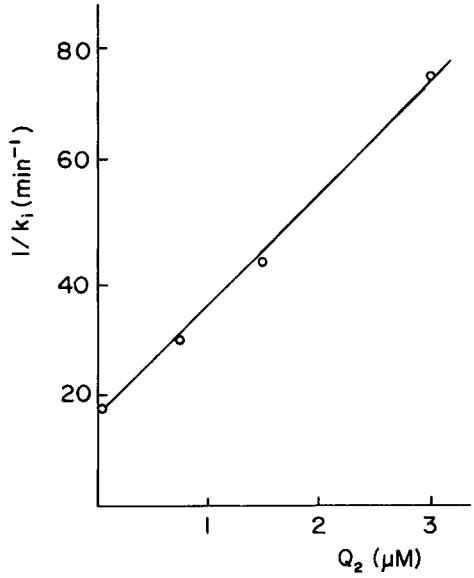

Fig. 3. The kinetics of pyridoxal phosphate-induced inactivation of the succinate-ubiquinone reductase in the presence of Q_2. The enzyme (10 μg/ml) was treated by 20 mM pyridoxal phosphate and assayed as described in Fig. 1. 20 mM succinate and various concentrations of Q_2 were added to the incubation medium before pyridoxal phosphate.

TABLE II

THE RELATIVE AFFINITIES OF THE REDUCED AND OXIDIZED SUCCINATE-UBIQUINONE REDUCTASE FOR THE QUINONES AND SOME SPECIFIC INHIBITORS

Experiments were done at 25°C, with 20 mM potassium phosphate, pH 8.0; for the experimental details, see Table I and Fig. 3. For E_{ox} 10 mM potassium fumarate was present; for E_{red} 20 mM potassium succinate was present. No protection against the pyridoxal phosphate-induced inactivation was observed for Q_0, and also not for Q_2 with E_{ox}. The apparent K_m and V_{max} for the carboxin-sensitive reduction of Q_0 were 4 μM and 70% of that measured with Q_2, respectively. The simple non-competitive with Q_2 and succinate inhibition with K_i of 1 μM was found in the catalytic assays for pentachlorophenol.

Substrate or inhibitor	K_d (μM)	
	E_{ox}	E_{red}
Q_2	–	0.3 [a]
Q_2H_2	> 5 [b]	0.9
Q_0	–	–
Carboxin	1.0	2.7
Thenoyltrifluoroacetone	62	45
Pentachlorophenol	2.2	1.0

[a] This value is taken from Ref. 13.

[b] The apparent complete protection was observed in the presence of 20 μM quinone; no kinetic analysis was possible, since Q_2H_2 undergoes autoxidation slowly in the presence of the enzyme.

Inactivation of the particles-bound succinate-ubiquinone reductase by pyridoxal phosphate

The results presented in the previous section and those reported earlier [12,13] show that the isolated enzyme reduced by succinate (E_{re}) interacts with both oxidized and reduced quinone, whereas no interaction between the oxidized enzyme (E_{ox}) and oxidized quinone was revealed by the approach employed in this study. The latter finding suggests that either E_{ox} does not interact with the oxidized quinone, or that the specific complex between E_{ox} and Q_2 exists being susceptible for pyridoxal phosphate attack as well as the quinone-depleted enzyme. It was therefore of interest to study an inactivating effect of pyridoxal phosphate on the particles-bound enzyme where the redox state of the natural acceptor (ubiquinone) can be easily varied simply by addition of succinate or malonate. As shown in Fig. 4 an inactivation of the particles-bound enzyme also

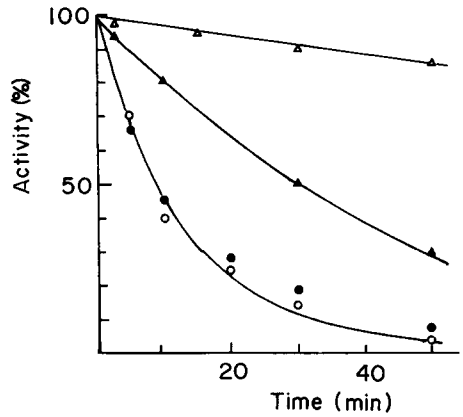

Fig. 4. Inactivation of the membrane-bound succinate-ubiquinone reductase by pyridoxal phosphate. The submitochondrial particles (8 mg/ml) were incubated as described in Fig. 1 with 30 mM pyridoxal phosphate; other additions were: (○) 0.5 mM malonate; (●) 20 mM succinate and 5 mM azide; (△) 0.5 mM malonate and 0.1 mM carboxin; (▲) 20 mM succinate and 50 μM Q_2. The succinate-ubiquinone reductase activity was determined as described in Fig. 1. 100% corresponds to the original activity of 0.9.

occurs in the presence of pyridoxal phosphate. An interesting point shown in Fig. 4 is that essentially the same rates of inactivation were observed in the presence of succinate plus azide (the enzyme and ubiquinone are reduced) or malonate (the enzyme and ubiquinone are oxidized). Carboxin protects the particle-bound enzyme against inactivation. Table III demonstrates that the inactivating effect of pyridoxal phosphate on the particle-bound succinate-ubiquinone reductase is due to the dissociation of the succinate dehydrogenase from the particles, as has been previously shown for the Triton X-100-solubilized enzyme [21]. It worthwhile mentioning that the solubilizing effect of pyridoxal phosphate (i.e., lysine or N-terminal aminoacid residues modification) on the particles is not specific for succinate dehydrogenase. When the submitochondrial particles treated with pyridoxal phosphate as described in Table III were sedimented in ultracentrifuge and the supernatant fraction was subjected to SDS-electrophoresis about 10 peptides including those with M_r values of 70000 and 30000 were recovered in the gel slabs. When the same procedure was performed in the presence of 100 μM carboxin the electrophoretic pattern of the supernatant was the same,

TABLE III

SOLUBILIZATION OF THE PARTICLE-BOUND SUCCINATE DEHYDROGENASE BY PYRIDOXAL PHOSPHATE

Submitochondrial particles (5 mg/ml) were incubated at 25°C for 1 h in a mixture containing 20 mM phosphate/1 mM malonate/0.1 mM EDTA (potassium salts, pH 8.0)/30 mM pyridoxal phosphate/0.1 mM carboxin (where indicated). The samples were cooled, centrifuged at 120000 × *g* for 1 h; the supernatants were collected and the sediments were suspended in the buffer containing 20 mM phosphate/10 mM succinate/0.1 mM EDTA (potassium salts)/0.2 M glycine (pH 7.8) (glycine was added to remove pyridoxal phosphate). The proper amounts of succinate and glycine were added to the supernatants to make the final concentrations 10 mM and 0.2 M, respectively. The activities were then determined as described in Materials and Methods and proper amounts of the supernatant fractions were taken for the electrophoretic analysis.

Samples	Treatment and the activities measured (μmol/min per mg)					
	succinate-ubiquinone reductase			succinate-PMS reductase		
	none	pyridoxal phosphate	pyridoxal phosphate + carboxin	none	pyridoxal phosphate	pyridoxal phosphate + carboxin
Submitochondrial particles	1.10	0.00	0.84	1.04	0.28	0.72
Sediment	0.96	0.00	0.60	0.84	0.00	0.62
Supernatant	0.00	0.00	0.00	0.02	0.32	0.10

except for the peptides corresponding to the succinate dehydrogenase subunits which were absent (the results are not shown).

Effect of pyridoxal phosphate treatment on the soluble succinate dehydrogenase

The data presented in this report together with earlier observations [21,35] strongly suggest the participation of lysil or N-terminal aminoacid residues in the maintaining of the succinate-ubiquinone reductase integrity. It is well known that the catalytic activity of succinate dehydrogenase within submitochondrial particle, complex II or soluble succinate-ubiquinone reductase [12] is quite stable, whereas being separated from the smaller peptides the two subunit succinate dehydrogenase rapidly loses the reductase activities relevant to the natural electron-transfer pathway [24,25,36,37]. Consequently the reconstitutive and ferricyanide reductase activities can be taken as a measure of the native conformation of the soluble succinate dehydrogenase. As shown in Fig. 5 the treatment of the soluble reconstitutively active

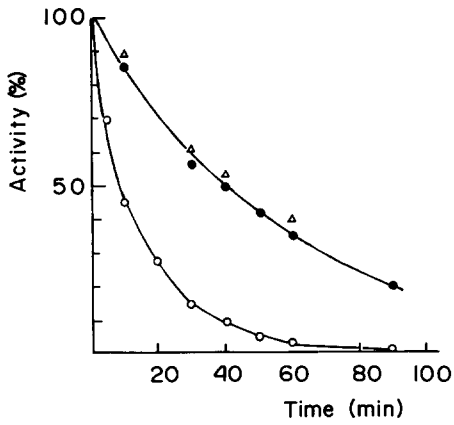

Fig. 5. The protective effect of pyridoxal phosphate on the soluble succinate dehydrogenase. (○) control (the succinate-ferricyanide reductase); (●) pyridoxal phosphate-treated enzyme (the succinate-ferricyanide reductase); (△) pyridoxal phosphate-treated enzyme (the reconstitution test). Succinate dehydrogenase (0.3 mg/ml) was incubated aerobically at 25°C for the time indicated on the abscissa in a mixture containing 0.1 phosphate/20 mM succinate/1 mM EDTA (potassium salts, pH 8.0)/15 mM pyridoxal phosphate (● and △). The samples from the mixture were withdrawn, mixed with equal volume of a buffer containing 10 mM succinate, 20 mM potassium phosphate, 0.2 M glycine, 1 mM EDTA (pH 7.4) and incubated for 5 min. The proper amounts of the samples were taken for the succinate-ferricyanide (150 μM) reductase assays. The suspension of alkali-treated Keilin-Hartree preparation [39] was then added to the samples (the final protein content was 3 mg/ml) and incubation was continued for 15 min. Q_2 (5 μM)-mediated DCIP (50 μM) reduction was then measured. The control (○) was treated exactly as described except for 0.2 M glycine was added to the pyridoxal phosphate-containing buffer before aerobic inactivation of the enzyme was started. 100% corresponds to the original activity of 10 (per mg of the soluble enzyme protein) in either assay.

succinate dehydrogenase with pyridoxal phosphate significantly protects the enzyme against aerobic inactivation with respect of both 'low K_m' ferricyanide reductase and the reconstitution test. Since there is strong interference between the absorbances due to ferricyanide and pyridoxal phosphate, the reactivity of the enzyme toward ferricyanide could not be directly measured in the presence of pyridoxal phosphate; however, the protective effect of pyridoxal phosphate in the aerobic preincubation mixture is evident.

Discussion

We have previously shown that the succinate-ubiquinone reductase solubilized and purified in the presence of Triton X-100 being rapidly diluted from the concentrated solutions appears as the carboxin-insensitive ferricyanide reductase, whereas it appears as the carboxin-sensitive ubiquinone reductase when added to the assay mixture from the diluted solutions [12,32]. It has been proposed that the slow equilibrium between two alternative states of the enzyme exists in the solution which is strongly dependent on the protein/detergent concentration. The results on different Q_2 sensitivity of the pyridoxal phosphate-induced inactivation in the diluted or concentrated system (Fig. 2, Table I) give direct support to this proposal. It appears that no ubiquinone binding site exists in the concentrated solution of the enzyme. It is worthwhile mentioning that due to the sensitivity limits many properties of the purified respiratory chain components (i.e., optical and ESR spectra, hydrodynamic behaviour, ligands binding) are usually studied in much more concentrated solutions than those used for the catalytic assays. An unusual behaviour of the Triton X-100-solubilized succinate-ubiquinone reductase [12,32], which is also evident from the results reported here, clearly shows that the great precautions should be taken when the data on the catalytic and structural properties of the respiratory chain components are comparatively analyzed.

The main purpose of this study was to quantitate the quinone-binding properties of the enzyme in different redox states. We have recently reported that the affinity of the dicarboxylate binding site of succinate dehydrogenase is strongly dependent on the redox state of the enzyme [22]. The results presented here (Table II) show that this is also true for the acceptor binding site. The observation on the specific interaction between E_{red} and Q_2H_2 is in agreement both qualitatively and quantitatively with our previous data on the kinetic competition between Q_2 and Q_2H_2 for the specific binding site [13]. Although the affinities for Q_2 and Q_2H_2 to the enzyme are altered by the redox state, no significant difference was observed for binding of thenoyltrifluoroacetone and other specific inhibitors to the reduced and oxidized enzyme. This finding agrees with the non-competitive (with respect of Q_2) inhibition of succinate-ubiquinone reductase by these inhibitors [12] and suggests that the different sites of the enzyme are involved in quinone and the inhibitors binding. Whatever the chemical mechanism of the quinone binding is, the data obtained suggest strong structural rearrangement of the enzyme upon the catalytic redox cycle.

It would not be judicious to apply directly the conclusions based on the experiments with artificial homolog of ubiquinone to the natural system containing Q_{10} as the acceptor. Thus, the data obtained with the submitochondrial particles merit some special discussion. The protective effect of carboxin taken together with the SDS electrophoresis data clearly indicate that essentially the same mechanism of pyridoxal phosphate inhibition of the particles-bound and soluble enzyme is operating. The basic difference is that in contrast to the soluble system the same kinetics of pyridoxal phosphate-induced inactivation is seen when endogenous ubiquinone and the enzyme are reduced or oxidized. In other words, no detectable protection against pyridoxal phosphate-induced dissociation by the natural ubiquinone was found. These results indicate that either the mechanisms of Q_2 and the natural ubiquinone binding are different, or, more likely, that in the natural system the enzyme ubiquinone binding site is not saturated. In fact, the latter seems to be expected, since much higher lipophylity of Q_{10} compared to Q_2 should result in a decrease of the affinity of quinone to the specific site formed by the protein moiety within highly nonpolar phospholipid bilayer.

The protective effect of pyridoxal phosphate on

aerobic inactivation of the soluble reconstitutively active succinate dehydrogenase seems to be a useful tool for practical purposes: since the formation of Shiff's base is readily reversible, pyridoxal phosphate can be easily removed just by dilution in the presence of proper amine. It also gives some clue on the chemical nature of strong binding between the succinate dehydrogenase and ubiquinone reactivity conferring proteins. It may be proposed that an amino group(s) of succinate dehydrogenase participate in electrostatic interaction with a carboxylic group(s) of the small peptide counterpart, thus making iron-sulfur cluster protected from deteriorating effect of oxygen. The analysis of the methods empirically found so far for a resolution of the succinate-ubiquinone reductase, i.e., use of chaotropic agents [38], alkaline treatment [39], solubilization by cyanide [40] and pyridoxal phosphate-induced dissociation [21] seems also support this hypothesis.

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