Redox Potentials and Quinone Reductase Activity of L-Aspartate Oxidase from *Escherichia coli*†

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ABSTRACT: L-Aspartate oxidase (EC 1.4.3.16) is a flavoprotein that catalyzes the first step in the *de novo* biosynthetic pathway to pyridine nucleotides both under aerobic and under anaerobic conditions. Despite the physiological importance of this biosynthesis particularly in facultative aerobic organisms, such as *Escherichia coli*, little is known about the electron acceptor of reduced L-aspartate oxidase in the absence of oxygen. In this report, evidence is presented which suggests that *in vitro* quinones can play such a role. L-Aspartate oxidase binds menadione and 2,3-dimethoxy-5-methyl-*p*-benzoquinone with K_d values of 11.5 and 2.4 μM, respectively. A new L-aspartate:quinone oxidoreductase acitivity is described in the presence and in the absence of phospholipids, and its possible physiological relevance is discussed. Moreover, considering the striking sequence similarity between L-aspartate oxidase and the highly conserved family of succinate-fumarate oxidoreductases, the redox properties of L-aspartate oxidase were investigated in detail. A value of −216 mV was calculated for the midpoint potential of the couple FAD/$FADH_2$ bound to the enzyme. This result perfectly explains why L-aspartate oxidase may be considered as a very particular fumarate reductase unable to use succinate as the electron donor.

L-Aspartate oxidase (EC 1.4.3.16) is a flavoenzyme containing 1 mol of noncovalently bound FAD per mole of protein (Nasu et al., 1982; White, 1982). The enzyme catalyzes the oxidation of L-aspartate to the corresponding imino acid under both aerobic and anaerobic conditions (Foster & Moat, 1980). In *Escherichia coli*, this reaction represents the first step in the biosynthesis of quinolinate by the quinolinate synthase complex. The molecule is subsequently converted to NAD via a metabolic sequence common to all organisms (White, 1982). Despite the physiological importance of this process, in particular in facultative aerobic organisms, like *E. coli*, little is known about the electron acceptor of reduced L-aspartate oxidase in the absence of oxygen. Very recently, we proposed that fumarate can play such a role *in vitro*; an L-asparate:fumarate oxidoreductase activity was observed, and Tedeschi et al. (1996) suggested that L-aspartate oxidase can be considered as a very particular soluble fumarate reductase, incapable of oxidizing succinate. This behavior is well in accordance with the observation of Ackrell et al. (1991), who suggest that the earliest fumarate-reducing systems were soluble forms with noncovalent FAD and the ability of contemporary fumarate reductase to oxidize succinate is the result of covalent attachment of FAD resulting in an increase in the redox potential of the coenzyme. Furthermore, a marked sequence similarity was described between L-aspartate oxidase and the flavoprotein subunit of fumarate reductase (FRD-A)[1] and succinate dehydrogenase (SDH-A) (Mortarino et al., 1996), but similarity with the other components of the fumarate reductase or succinate dehydrogenase complexes has received little attention with regard to composition, structural organization, and interaction with quinones. It is well-known, indeed, that fumarate reductase (as well as succinate dehydrogenase) is a complex of four nonidentical subunits A, B, C, and D; FRD-A and -B form the catalytic domain, whereas FRD-C and -D are small hydrophobic subunits that are essential for attachment of the catalytic components to the membranes and for the electron transfer through the interaction with quinones at two different sites (Westemberg et al., 1993). Both SDH and FRD complexes transfer electrons from the donor to the acceptor via these multiple domains, but the electron flow is in the opposite direction. FRD transfers electrons from reduced quinol like menadiol ($E° = -70$ mV) to fumarate ($E° = 30$ mV), whereas SDH transfers electrons from succinate ($E° = 30$ mV) to quinone like ubiquinone ($E° = 100$ mV) (Ackrell et al., 1991). Therefore, FRD contains, in general, centers with lower standard redox potentials compared with that of SDH. It was obviously very interesting to compare these two enzymes with L-aspartate oxidase, described as a novel fumarate reductase and a good model for this class of proteins (Tedeschi et al., 1996). In this study, the redox properties of L-aspartate oxidase are described both in the presence and in the absence of the products of the reductive and the oxidative half-reaction. Moreover, we suggest that quinones can bind at or close to the active site of the enzyme and a new L-aspartate:quinone oxidoreductase activity is described under anaerobic conditions. The effect of phospholipids and

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[1] Abbreviations: FRD, fumarate reductase; SDH, succinate dehydrogenase; TMNO, trimethylamine *N*-oxide; TN, turnover number.

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membranes vesicles in the assay mixture is also described in detail, and the results are discussed keeping in mind the possible physiological relevance of this activity.

MATERIALS AND METHODS

L-Aspartate oxidase overexpressed in *E. coli* was purified according to the procedure described (Mortarino et al., 1996), and the total protein content was determined from the known extinction coefficient using an $\epsilon_{452\ nm}$ of 11 600 M^{-1} cm^{-1}. Samples for anaerobic experiments were prepared by alternate evacuation and flushing with oxygen-free argon (Foust et al., 1969).

Xanthine oxidase was purchased from Sigma. All other chemicals were of analytical reagent grade. 5-Deazaflavin-3-sulfonate was a generous gift of V. Massey (The University of Michigan, Ann Arbor, MI).

Absorption spectra were measured with a Jasco recording spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer.

Determination of the Redox Potential of L-*Aspartate Oxidase*. The redox potential for the flavoprotein was determined at 25 °C in 8 mM imidazole at pH 7.0 by a spectrophotometric method employing a reducing system of xanthine and xanthine oxidase and a suitable mediator such as benzylviologen (Massey, 1990). When the redox potential was determined in the presence of iminoaspartate, the imino acid was produced *in situ* by using an enzymatic system consisting of D-aspartate/D-aspartate oxidase as reported by Mortarino et al. (1996). D-Aspartate (300 μM) was reacted with D-aspartate oxidase in the presence of 15 μM L-aspartate oxidase. The production of the enzyme−iminoaspartate complex was followed at 500 nm until saturation, and gel filtration on a PD-10 column was carried out in order to eliminate the excess of reagents. The amount of the complex recovered after elution was quantitated from the characteristic ratio between the absorbance at 500 and 452 nm.

Preparation of Membrane Vesicles from E. coli. Membrane vesicles from *E. coli* cells (strain BL21) were obtained by lysing the cells with 10 mM EDTA and 0.5 mg/mL lysozyme at pH 6.8 (Kabak, 1971). After incubation for 30 min at room temperature, the material was centrifuged at 22500*g* for 30 min at 4 °C. The precipitate was resuspended with 0.1 M potassium phosphate (pH 6.6), 20% sucrose, and 20 mM $MgSO_4$, and it was homogenized in the presence of 5 mg/mL DNase and 27 μg/mL RNase. After incubation at 37 °C for 15 min with vigorous swirling, the lysates were centrifuged at 17800*g* for 30 min and the pellet was resuspended in 0.1 M potassium phosphate (pH 6.6) containing 10 mM EDTA at 0 °C. The suspention was centrifuged at 800*g* for 30 min, and the procedure was repeated three times using the precipitate resuspended in the same buffer. The supernatants were collected, carefully decanted by centrifugation at 45000*g* until they were clear, and discarded. The remaining precipitate was resuspended in 0.1 M potassium phosphate (pH 7.0) and added to the assay or incubation mixture as specified below. Alternatively, vesicles were obtained by sonication according to Futai (1974). Two grams of wet cells was suspended in 80 mL of 0.01 M Tris-HCl (pH 7.4), containing 10 mM $MgCl_2$ and 10 μg/mL DNase and RNase, and sonicated for 7 min. After centrifugation at 800*g* for 10 min, the supernatant was centrifuged twice at 30000*g* for 30 min. The pellet was suspended in 50 mM Hepes at pH 8 and used in the experiments as specified below.

Photoreduction of L-*Aspartate Oxidase*. Photoreduction of L-aspartate oxidase was achieved by irradiating the protein under anaerobic conditions in the presence of 15 mM EDTA, 1 μM 5-deazaflavin-3-sulfonate as catalyst, and 5 μM methylviologen at 25 °C in 50 mM Tris-HCl (pH 8.0) and 20% glycerol (Massey et al., 1978).

L-*Aspartate:Quinone Oxidoreductase Activity*. The reaction mixture contained various amounts of quinone and L-aspartate in a total volume of 3 mL in 50 mM Tris-HCl (pH 8.0) and 20% glycerol. After anaerobiosis, 10 μL of 102 μM L-aspartate oxidase was added from the sidearm of an anaerobic cuvette, and the reaction was carried out at 25 °C following the reduction of quinone.

When cardiolipin and phosphatidylethanolamine were present in the assay mixture, they were solubilized in methanol and chloroform and 50 μL of each solution was incubated with L-aspartate oxidase in the presence of excess FAD before the other components of the assay mixture were added.

Determination of K_d for 2,3-Dimethoxy-5-methyl-p-benzoquinone by Ultrafiltration. L-Aspartate oxidase (19 μM) in 1 mL of 50 mM Hepes at pH 8.0 was incubated with 100 μM 2,3-dimethoxy-5-methyl-*p*-benzoquinone and concentrated to about 200 μL by centrifugation with an Amicon Centricon 30 instrument at 4 °C. The exact volume and the spectrum of the concentrated material and the filtrate were measured, and the K_d was calculated from the amount of free quinone determined by the absorbance at 410 and 450 nm (=1125 and 825 M^{-1} cm^{-1}, respectively). The result was confirmed by diluting the concentrated material with fresh buffer and repeating the concentration and measurement steps.

Sequence Analysis Studies. Sequence analysis studies were performed using the PC-GENE program (Intelligenetics, Campbell, CA), and exhaustive database searches were obtained on the Swiss-Prot Data Bank using the same program.

NMR Studies. The ^{1}H-NMR spectra of 2,3-dimethoxy-5-methyl-*p*-benzoquinone dissolved in D_2O were recorded at 270 MHz with an AH-Bruker spectrometer. The experiments were performed at 23 °C and pH 8.0. The enzyme titration was implemented by adding measured amounts of concentrated enzyme, dissolved in D_2O, to the NMR tube containing the quinone. HDO was used as internal reference frequency standard.

RESULTS

Determination of Redox Potentials of L-*Aspartate Oxidase*. To measure the potential of the FAD/$FADH_2$ couple in L-aspartate oxidase, a reductive titration was carried out in the presence of anthraquinone-2,6-disulfonate ($E° = -0.215$ V) (Figure 1) according to the method of Massey (1990). A 45 μL sample of 1 mM dye was added from the sidearm of an anaerobic cuvette to 1 mL of 19 μM L-aspartate oxidase in 8 mM imidazole (pH 7.0), 0.5 mM xanthine, 6 mM EDTA, and 5 μM benzylviologen at pH 7.0 and 25 °C. Milk xanthine oxidase (2.38×10^{-8} M) was added to start the reaction. The amounts of oxidized and reduced dye and L-aspartate oxidase were quantitated at 330 nm (corrected for the contribution of the coenzyme) and 460 nm (corrected

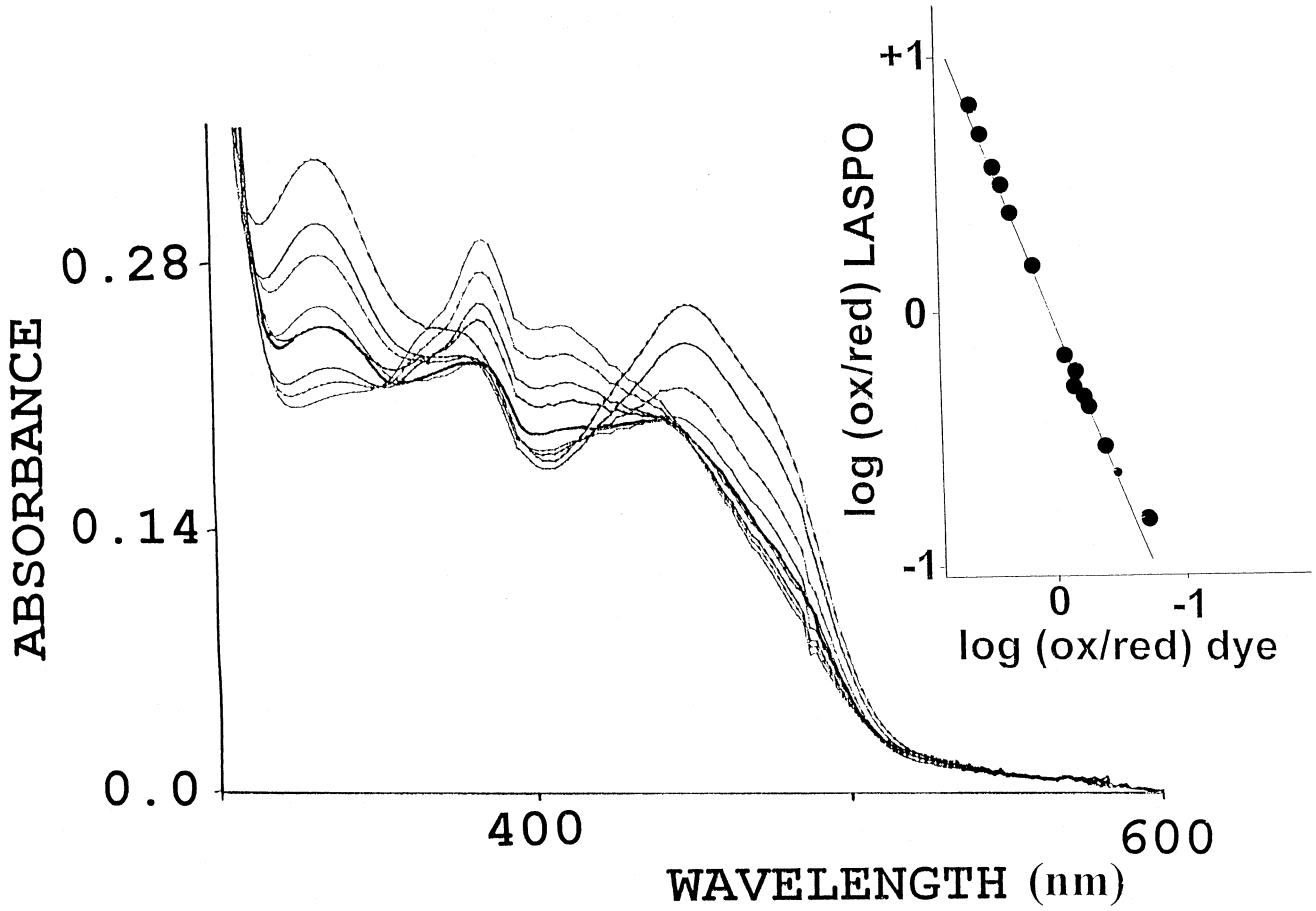


FIGURE 1: Anaerobic determination of the L-aspartate oxidase redox potential by equilibration with anthraquinone-2,6-disulfonate. L-Aspartate oxidase in 8 mM imidazole buffer (pH 7.0) and 20% glycerol, at 25 °C, containing 6 mM EDTA, 0.5 mM xanthine, 5 μM benzylviologen, and 30 μM anthraquinone-2,6-disulfonate was incubated under anaerobic conditions with xanthine oxidase. (Inset) Data obtained while monitoring reduction of the dye at 330 nm, corrected for the contribution of FAD, and reduction of FAD at 460 nm, corrected for the contribution of the dye.

Table 1: Redox Potentials for L-Aspartate Oxidase at 25 °C and pH 7.0

indicator dye	E_{m_7} indicator (V)	E_{m_7} for L-aspartate oxidase (V)
anthraquinone-2,6-disulfonate	−0.215	−0.216
cresyl violet acetate	−0.166	−0.216
janus green	−0.226	−0.222
anthraquinone-2,6-disulfonate + 0.75 mM photoreduced menadione	−0.215	−0.217
anthraquinone-2,6-disulfonate + iminoaspartate	−0.215	−0.216
anthraquinone-2-sulfonate + 5 mM NAD	−0.225	−0.216

for the contribution of the dye), respectively. According to the method of Minnaert (1965), the log(ox/red) for the anthraquinone-2,6-disulfonate was plotted *versus* the log-(ox/red) for the enzyme-bound FAD (Figure 1, inset). The plot gives a 1 unit slope and a value of −0.216 V for the FAD/$FADH_2$ potential (Table 1). A similar titration was carried out in the presence of cresyl violet acetate ($E°$ = −0.166 V) (Banny & Clark, 1950) which undergoes a loss of absorbance at 590 nm during reduction (Figure 2). The reduction of L-aspartate oxidase was calculated from the decrease in absorbance at 452 nm corrected for the contribution of the dye. The potential for the FAD/$FADH_2$ couple was found to be −0.216 V (Table 1), in excellent agreement with the value from the anthraquinone-2,6-disulfonate titration. From the Minnaert plot (Figure 2, inset), a 1 unit slope was calculated, as expected for equilibrium between a two-electron acceptor dye and a two-electron donor without semiquinone formation. The same result was obtained with 37 M janus green as the electron dye (E = −0.266 V) (Banny & Clark, 1950) by following the reduction of the indicator at 620 nm and the reduction of L-aspartate oxidase at 460 nm (Table 1). As previously reported (Tedeschi et al., 1996), 40% of blue semiquinone is formed and kinetically stabilized upon photoreduction. According to Clark (1960), this corresponds to a very small separation of the two redox potentials of FAD/$FADH^{\bullet}$ (E_{m_1}) and $FADH^{\bullet}$/$FADH_2$ (E_{m_2}) in the enzyme. From the Nerst equation, ($E_{m_1} - E_{m_2}$) was calculated to be −0.0147 at 25 °C and pH 7.0, and from the midpoint potential of −0.216 V, E_{m_1} and E_{m_2} can be predicted to be −0.223 and −0.209 V, respectively, suggesting that FAD bound to L-aspartate oxidase is reduced and reoxidized in one two-electron step without semiquinone formation (Tedeschi et al., 1995). No changes in the redox potential were observed when the redox titration with anthraquinone-2,6-disulfonate was repeated in the presence of 0.75 mM photoreduced menadione or iminoaspartate (Table 1). As shown in Figure 3, the same result was obtained in the presence of 5 mM NAD which is reported to be an inhibitor of the enzyme (Nasu et al., 1982). From the shift in midpoint potential of FAD (−0.207 V) (Massey, 1990) upon binding to the enzyme (−0.216 V) and the known dissociation constant of FAD from the oxidized protein (K_{ox} = 0.67 μM)

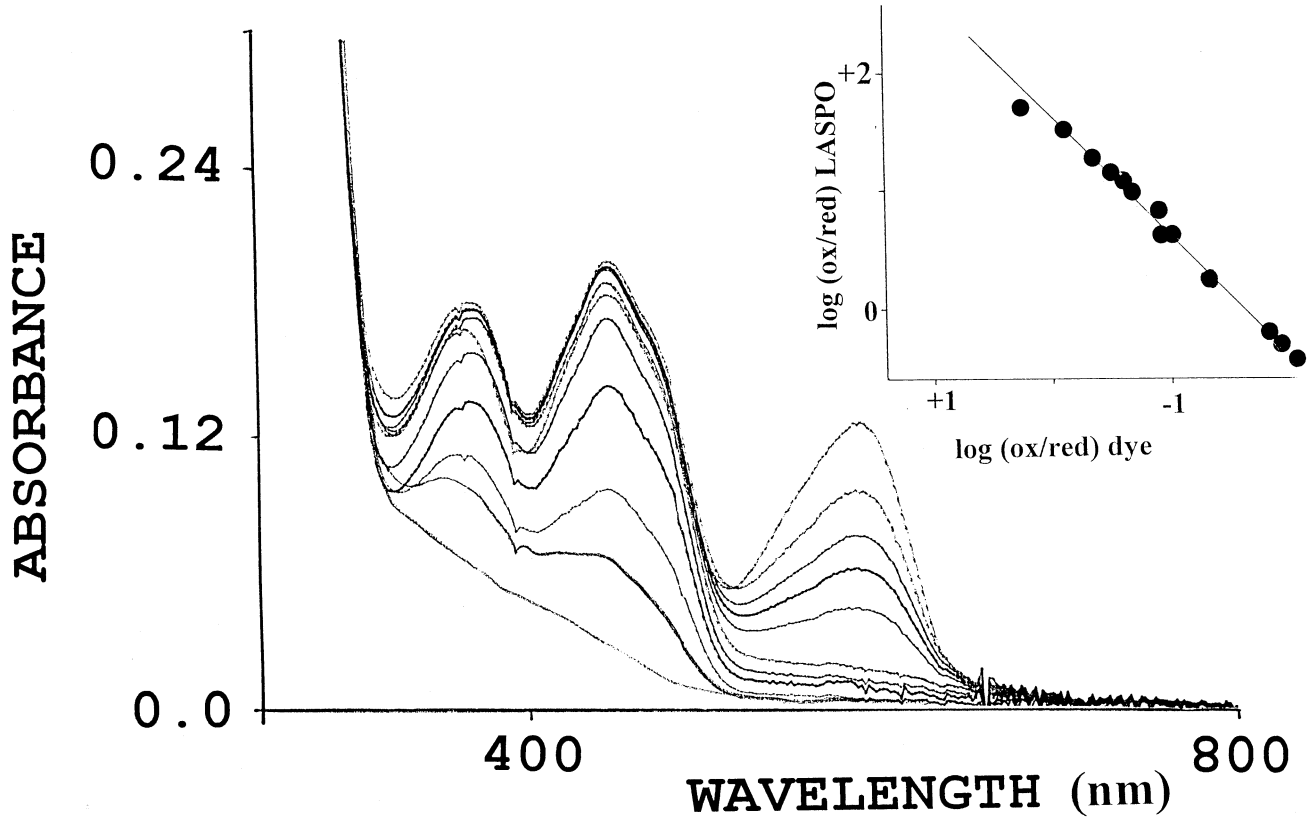


FIGURE 2: Anaerobic determination of the L-aspartate oxidase redox potential by equilibration with cresyl violet acetate. L-Aspartate oxidase in 8 mM imidazole buffer (pH 7.0) and 20% glycerol, at 25 °C, containing 6 mM EDTA, 0.5 mM xanthine, 5 μM benzylviologen, and 33 μM cresyl violet acetate was incubated under anaerobic conditions with xanthine oxidase. (Inset) Data obtained while monitoring reduction of the dye at 590 nm and reduction of FAD at 460 nm corrected for the contribution of the dye.

(Mortarino et al., 1996), a value of 1.35 μM for K_{red} can be calculated using the following equation (Clark, 1960):

$$\Delta E = RT/nF \times \ln(K_{ox}/K_{red}) \quad (1)$$

These data and the data reported in Table 1 show a marked difference between L-aspartate oxidase and fumarate reductase or succinate dehydrogenase which covalently bind FAD, resulting in an increase in the redox potential of the coenzyme which clearly lacks in L-aspartate oxidase.

Binding of Naphtho- and Benzoquinones to L*-Aspartate Oxidase*. Succinate and fumarate are very well-known substrates for the flavoprotein subunits of the fumarate reductase and succinate dehydrogenase complexes. As previously reported, these two molecules bind to L-aspartate oxidase at or close to the active site of the enzyme, causing perturbation of the visible absorption spectrum of FAD (Tedeschi et al., 1996). When 20 μM L-aspartate oxidase was titrated with menadione in 50 mM Tris-HCl at pH 8.0 and 25 °C, the same spectral perturbations were observed (data not shown). A K_d of 11.5 μM was calculated from a Benesi−Hildebrand plot (Benesi & Hildebrand, 1949) of the differential spectra obtained in the titration experiments. However, it should be underlined that, as already observed for fumarate or succinate, additional small spectral perturbations were registered at ligand concentrations of higher than 1 mM, but no further studies were attempted because of the development of turbidity. In the case of 2,3-dimethoxy-5-methyl-*p*-benzoquinone, the spectral absorbance of the molecule in the range of 300−500 nm ruled out the possibility of checking the binding by titration experiments. For this reason, the K_d was determined by ultrafiltration and calculated to be 2.4 μM. Moreover, ^{1}H-NMR studies of the quinone in the absence and in the presence of L-aspartate oxidase were attempted in order to evaluate the k_{off} for the binding to the enzyme. Figure 4a shows the ^{1}H-NMR spectrum of 5 mM 2,3-dimethoxy-5-methyl-*p*-benzoquinone prepared in D_2O in the presence of 10 mM KP_i at pH 8.0. Figure 4b shows the expanded region of the spectrum containing the methoxy resonances 3 and 3′ at 3.87 and 3.88 ppm, respectively. Upon addition of L-aspartate oxidase, the line resonances are selectively broadened but there are only very small changes in chemical shifts (Figure 4a). This is characteristic of a fast exchange between the bound and the free states (Craik & Higgins, 1989). Association/dissociation rate constants were derived from the ligand titration data, using 0.5, 1.2, 5, 10, 15, 20, and 50 μM L-aspartate oxidase, by monitoring the progressive broadening of the methoxy resonances of peak 3 and 3′. k_{off} was determined from the slope of the plot of the excess line widths *versus* the fraction of bound quinone (Pb) under conditions where the free ligand is in excess and Pf $\approx$ 1 (Craik & Higgins, 1989; Ehrlich & Colman, 1985) and calculated to be 1.75×10^4 s^{-1} (inset of Figure 4a). From this value and the corresponding K_d, k_{on} was calculated to be 7.8×10^9 M^{-1} s^{-1}, which indicates that the ligand-binding kinetics is close to being diffusion-controlled, suggesting a favorable entropy factor for the quinone penetrating into the substrate binding site (De Marco et al., 1987).

Protection by Menadione in Chemical Modification Studies. As reported by Seifer et al. (1990), treatment of L-aspartate oxidase with iodoacetic acid (molar ratio of reagent:protein = 255:1) or tetranitromethane (molar ratio

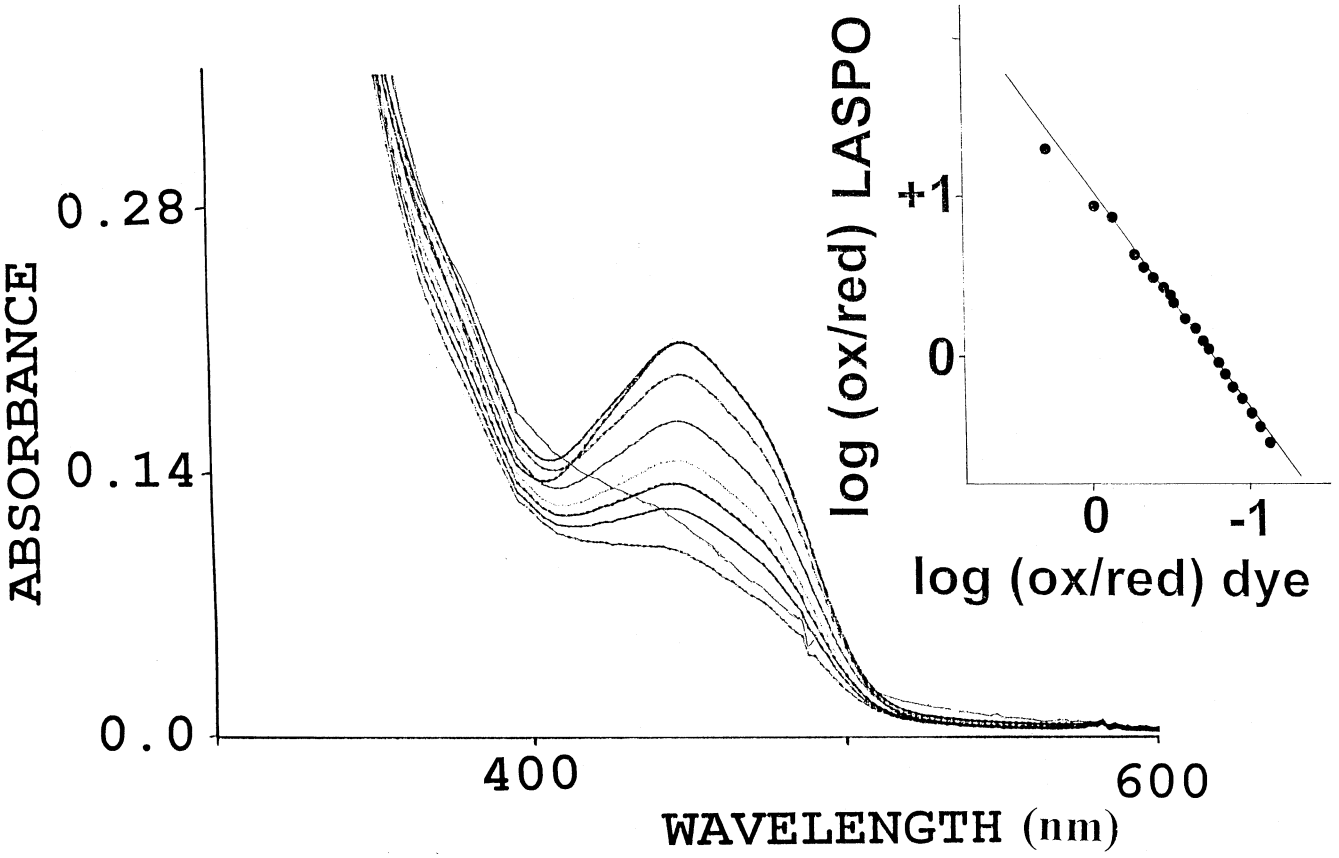


FIGURE 3: Anaerobic determination of the L-aspartate oxidase redox potential by equilibration with anthraquinone-2,6-disulfonate in the presence of 5 mM NAD. L-Aspartate oxidase in 8 mM imidazole buffer (pH 7.0) and 20% glycerol, at 25 °C, containing 6 mM EDTA, 0.5 mM xanthine, 5 μM benzylviologen, 5 mM NAD, and 30 μM anthraquinone-2,6-disulfonate was incubated under anaerobic conditions with xanthine oxidase. (Inset) Data obtained while monitoring reduction of the dye at 380 nm, corrected for the contribution of FAD, and reduction of FAD at 460 nm, corrected for the contribution of the dye.

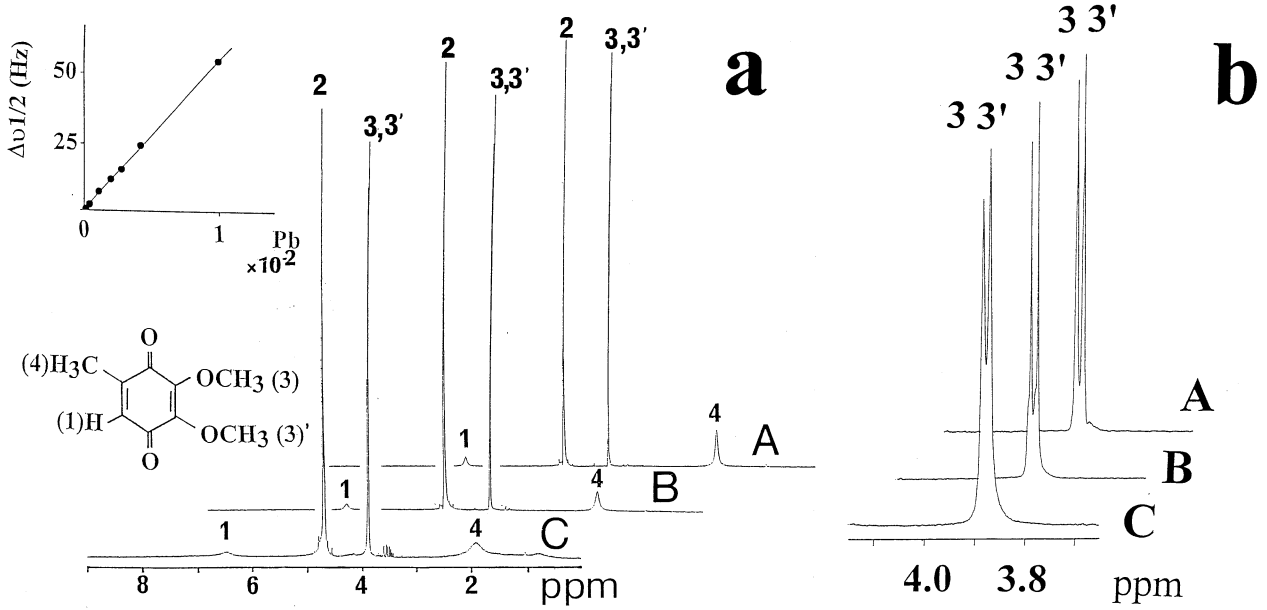


FIGURE 4: (a) ^{1}H-NMR spectra of 2,3-dimethoxy-5-methyl-*p*-benzoquinone in 10 mM KP_i at pH 8.0 prepared in D_2O in the absence (A) and in the presence of 5 μM (B) and 50 μM (C) L-aspartate oxidase. (Inset) Broadening of peaks 3 and 3′ (average values) as a function of the proportion of quinone bound (Pb). The broadening ($\Delta\nu_{1/2}$) is the difference between the measured line widths at the half-maximum amplitude of the quinone−enzyme complex and of free quinone in the absence (A) and in the presence of 5 μM (B) and 50 μM (C) L-aspartate oxidase. (b) Expanded spectrum of peaks 3 and 3′ in the absence of L-aspartate oxidase.

of reagent:protein = 3:1) results in a rapid loss of L-aspartate oxidase activity. When the same experiments were carried out in the presence of 4 mM menadione in the incubation mixture, the inactivation of the enzyme was sensibly retarded. Rates of 0.0018 and 0.00123 min^{-1} were calculated for the inactivation by iodoacetic acid and tetranitromethane, respectively. These values should be compared with those obtained in the absence of menadione (0.0063 and 0.0462

Table 2: Kinetic Parameters for L-Aspartate:Quinone Oxidoreductase Activity of L-Aspartate Oxidase under Anaerobic Conditions

electron acceptor	K_m a[a] (mM)	K_m b[a] (mM)	TN (min^{-1})	TN/K_m[b] (min^{-1} M^{-1})
2,3-dimethoxy-5-methyl-*p*-benzoquinone	16.6	0.5	100	2.0×10^5
menadione	22	0.15	67	4.47×10^5
oxygen[b]	5.2	0.25	156	6.24×10^5
fumarate[b]	2.7	2.5	333	1.33×10^5

[a] K_m a and K_m b refer to the K_m values of L-aspartate and the electron acceptor, respectively. [b] Tedeschi et al. (1996).

min^{-1}) and suggest a marked protection exerted by the quinone toward inhibition.

L-Aspartate:Quinone Oxidoreductase Activity of L-Aspartate Oxidase. Photoreduction of L-aspartate oxidase was achieved by irradiating the protein in the presence of EDTA, methylviologen, and 5-deazaflavin-3-sulfonate as the catalyst under strictly anaerobic conditions. Completely photoreduced L-aspartate oxidase was fully reoxidized by adding, from the sidearm of an anaerobic cuvette, either 990 μM benzoquinone, 976 μM menadione, 995 μM 2,3-dimethoxy-5-methyl-*p*-benzoquinone, 380 μM coenzyme Q_2, or 18 μM cytochrome *c*. These results prompted us to further investigate the possible role of quinones as electron acceptors for the enzyme. Steady state analyses were performed using L-aspartate as the electron donor and 2,3-dimethoxy-5-methyl-*p*-benzoquinone or menadione as the electron acceptors in the absence of oxygen. These compounds were chosen as more hydrophilic analogues of the physiological naphtho- and benzoquinones in *E. coli* (ubiquinone Q_8 and vitamine K_2, respectively) which are almost insoluble in an aqueous medium. In the presence of menadione, 5% ethanol was added to the assay mixture in order to avoid substrate precipitation. The study was performed by following the decrease in absorbance of oxidized quinones at 25 °C and pH 8.0, and the linearity of the assay in these conditions was carefully verified over a wide range of L-aspartate oxidase concentrations (0.05−2 μM). Quinone reduction was followed at 480 and 360 nm with extinction coefficients of 250 and 2266 M^{-1} cm^{-1} using 2,3-dimethoxy-5-methyl-*p*-benzoquinone and menadione, respectively. In both cases, the parallel pattern of the double-reciprocal plot is characteristic of a ping-pong system as previously described for L-aspartate oxidase reacting with fumarate (Tedeschi et al., 1996). The steady state data shown in Table 2 may be described by a general equation of the form (Segel, 1975)

$$e/v = \phi_0 + \phi_A[A] + \phi_B[B]$$

where [A] and [B] are L-aspartate and electron acceptor concentrations, respectively.

Comparison of these values with the corresponding data obtained in the presence of oxygen (Table 2) suggests that quinones, as well as fumarate, may replace oxygen under anaerobic conditions. On the contrary, no activity was observed using 10 mM KNO_3, 10 mM $NaNO_2$, or 10 mM TMNO.

As a way of confirming the kinetic mechanism proposed above, an alternative product like succinate was added to the assay mixture using 2,3-dimethoxy-5-methyl-*p*-benzoquinone as the electron acceptor. Succinate is the only

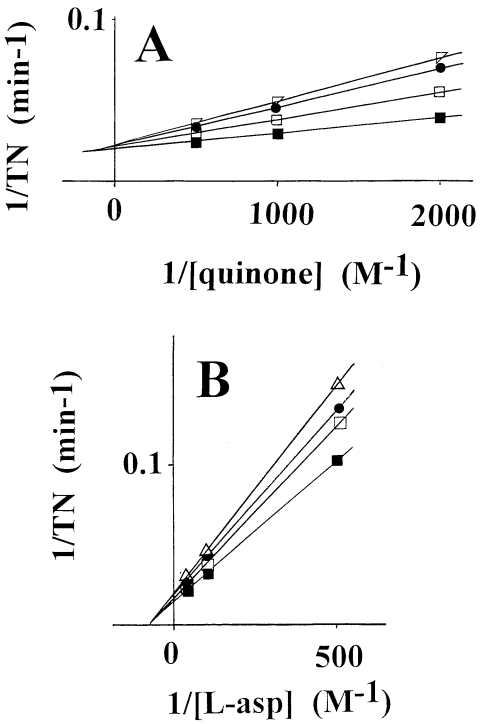
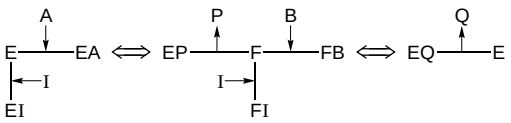


FIGURE 5: Steady state data for L-aspartate:2,3-dimethoxy-5-methyl-*p*-benzoquinone oxidoreductase activity in the presence of succinate. (A) Double-reciprocal plot, at the L-aspartate concentrations shown, using different succinate concentrations and 1 mM quinone: (■) 0 mM, (□) 1 mM, (●) 5 mM, and (rotated triangle) 10 mM. (B) Double-reciprocal plot, at the quinone concentrations shown, using different succinate concentrations and 10 mM L-aspartate: (■) 0 mM, (□) 1 mM, (●) 5 mM, and (△) 10 mM. In panels A and B, the value corresponding to 0.1 mM succinate is omitted for clarity.

product of fumarate reduction catalyzed by L-aspartate oxidase in the presence of L-aspartate and it binds to the oxidized enzyme with a K_d of 0.24 mM (Tedeschi et al., 1996). Moreover, the bindings of succinate, fumarate, and iminoaspartate to the enzyme are mutually exclusive. Figure 5A shows the double-reciprocal plot for L-aspartate as the varied substrate at a fixed concentration of quinone (1 mM). The plots of quinone as the varied substrate with 10 mM L-aspartate are symmetrical with respect to those shown in Figure 5A (Figure 5B). This pattern is expected in a ping-pong system if succinate combines with both oxidized and reduced enzyme according to the following reaction:



where E = the oxidized enzyme, A = L-aspartate, B = 2,3-dimethoxy-5-methyl-*p*- benzoquinone, F = the reduced enzyme, P = iminoaspartate, Q = reduced quinone, and I = succinate.

The inhibition constants K_{i_a} and K_{i_b} were calculated from the slope 1/*A* or 1/*B versus* [I] replots (K_{i_a} = 46 mM and K_{i_b} = 12 mM), whose patterns suggest that succinate and quinones may not bind simultaneously to the enzyme irrespective of whether the binding site for these ligands is the same or different (Segel, 1975).

Absence of Hydroquinone Dehydrogenase Activity of L-Aspartate Oxidase. Generally, many redox proteins that use quinone as an electron carrier have two quinone/quinol

binding sites, irrespective of whether the quinone redox mechanisms are the same or different. Fumarate reductase from *E. coli* (complex II) was recently proposed to have separate sites for interaction with reduced and oxidized quinones (Westemberg et al., 1993). An electron transfer mechanism similar to that in the photosynthetic reaction center appears to be operating at these quinone redox sites. The primary quinone is always tightly bound and acts as one-electron donor, while the secondary quinone, doubly reduced by the enzyme, is a two-electron, two-proton redox component. To better characterize the fumarate reductase behavior of L-aspartate oxidase, 2-hydroxy-1,4-naphthoquinol ($E_m = -0.137$ V, $E°$ for the quinone/quinol radical couple $= -0.46$ V) (Buffinton et al., 1989) was used in this study as the electron donor for L-aspartate oxidase. 2-Hydroxy-1,4-naphthoquinol (10 mM) was reduced by a stoichiometric amount of dithionite under strictly anaerobic conditions, and 10 mM fumarate was added from the sidearm of an anaerobic cuvette. Starting the reaction by adding 0.53 μM enzyme at 25 °C, no activity was detected even after incubation for 2 h. However, after the addition of L-aspartate, the enzyme catalyzed the reduction of fumarate. On the contrary, when oxidized 2-hydroxy-1,4-naphthoquinone (1 mM) was used in the presence of 10 mM L-aspartate as the electron donor, the reduction of quinone was observed and a TN of 2 min^{-1} was calculated by the decrease in absorbance at 454 nm. Furthermore, L-aspartate oxidase activity toward 2,3-dimethoxy-5-methyl-*p*-benzoquinone, menadione, and fumarate is unaffected by the presence of 20 μM 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide which is reported to be a strong inhibitor of the hydroquinone oxidase activity in fumarate reductase and succinate dehydrogenase but does not change fumarate and quinone reduction (Ackrell et al., 1991).

Enzymatic Reduction of Quinones Included in the Membrane of Liposomes. Liposomes were prepared by extrusion of a mixture of 95% egg lecithin and 5% $CHCl_3$ in 50 mM Tris-HCl (pH 8.0), 20% glycerol, and different amounts of menadione or coenzyme Q_7 at 25 °C. The reaction could be followed spectrophotometrically under strictly anaerobic conditions at an L-aspartate concentration of 10 mM. The rate was calculated from the decrease in absorbance at 276 nm using Q_7 and at 360 nm in the presence of menadione, with extinction coefficients of 12600 and 2266 M^{-1} cm^{-1}, respectively. The data clearly indicate that even a water insoluble molecule such as Q_7 can be reduced by L-aspartate oxidase if incorporated in the membrane of liposomes (K_m apparent $= 0.257$ mM, TN $= 5$ min^{-1}). Moreover, the rate of the reaction using menadione is the same irrespective of whether the compound is incorporated into the phospholipid bilayer or not, emphasizing the ability of L-aspartate oxidase to directly interact with hydrophobic quinones such as menadione (K_m apparent $= 10$ μM, TN $=17$ min^{-1}). When assayed in the presence of 500 μg of cardiolipin or 500 μg of phosphatidylethanolamine, this quinone reductase activity is not increased. The same result was obtained using oxygen as the electron acceptor, suggesting that zwitterionic and negatively charged lipids do not activate the enzyme either under anaerobic or under aerobic conditions. The L-aspartate: oxidase, L-aspartate:fumarate oxidoreductase, and L-aspartate: quinone oxidoreductase activities of L-aspartate oxidase were also checked in the presence of membrane vesicles obtained by lysing *E. coli* cells with EDTA and lysozyme or by sonication. Vesicles (100 or 800 μg) were incubated with 0.5 μM enzyme in 0.1 M phosphate buffer at pH 7.0 and 25 °C before starting the reaction with 10 mM L-aspartate. Otherwise, the vesicles were added to the assay mixture during turnover in order to minimize any inhibitory effect. In both cases, indeed, the presence of lipids does not affect enzyme activity. Finally, 2.5 nmol of L-aspartate oxidase was added to 800 μg of isolated membrane vesicles in 200 μL of 0.1 M phosphate buffer at pH 7.0 and 37 °C. Following dilution to 2 mL with the same buffer and centrifugation at 45000*g* for 30 min, a very weak binding of L-aspartate oxidase to membrane vesicles was observed.

DISCUSSION

Redox Properties of L-Aspartate Oxidase. From the present determination, a midpoint potential for the enzyme-bound FAD can be calculated to be -0.216 V at pH 7.0. This value is not very different from that of the free flavin ($E° = -0.207$ at pH 7.0) and corresponds to a ratio of binding constants of 0.5 with the oxidized flavin bound a little more tightly to the apoenzyme than reduced FAD. This results in thermodynamically more favorable oxidation of the coenzyme, in good agreement with recent observations which suggest that the reduction of L-aspartate oxidase by L-aspartate is the rate-limiting step in catalysis (unpublished results). Furthermore, the value of -0.216 V for the couple $FAD/FADH_2$ bound to the enzyme can perfectly explain the lack of L-aspartate oxidase reactivity using succinate as the electron donor. In fact, while the possibility exists that succinate, and/or the enzyme, changes its redox potential after binding, it is more likely that the thermodynamic barrier against electron transfer between this molecule and FAD cannot be circumvented. This result represents a major difference between L-aspartate oxidase and FRD-A and SDH-A which covalently bind FAD, resulting in an increase in the redox potential of the coenzyme (Ackrell et al., 1991).

Another different behavior between these two complexes and L-aspartate oxidase is proposed considering the very small separation between E_{m_1} and E_{m_2} potentials for the FAD bound to the latter enzyme. The data suggest that the coenzyme is reduced and reoxidized in a single two-electron step without semiquinone formation, in agreement with the results obtained using 2-hydroxy-1,4-naphthoquinone as the electron acceptor under anaerobic conditions. This quinone, indeed, has a very low redox potential for the quinone/quinone radical couple ($E° = -460$ V), and it is reported to cycle only from the oxidized to the completely reduced form. In the presence of L-aspartate, the molecule is an oxidant of L-aspartate oxidase, arguing against a single electron transfer mechanism for the enzyme.

The presence of reduced menadione or iminoaspartate has no effect on the midpoint potential of the enzyme-bound FAD, suggesting that the electron transfer to and from the enzyme is not controlled significantly by the product of the reductive and the oxidative half-reaction, despite the relatively low dissociation constants for the binding of the two molecules (11.5 μM for menadione and 1.4 μM for iminoaspartate). Finally, the redox properties of L-aspartate oxidase were analyzed in the presence of 5 mM NAD, which is the final product of the metabolic pathway for the *de novo* biosynthesis of pyridine nucleotides. Also in this case, the presence of the nucleotide does not modulate the redox properties of the enzyme.

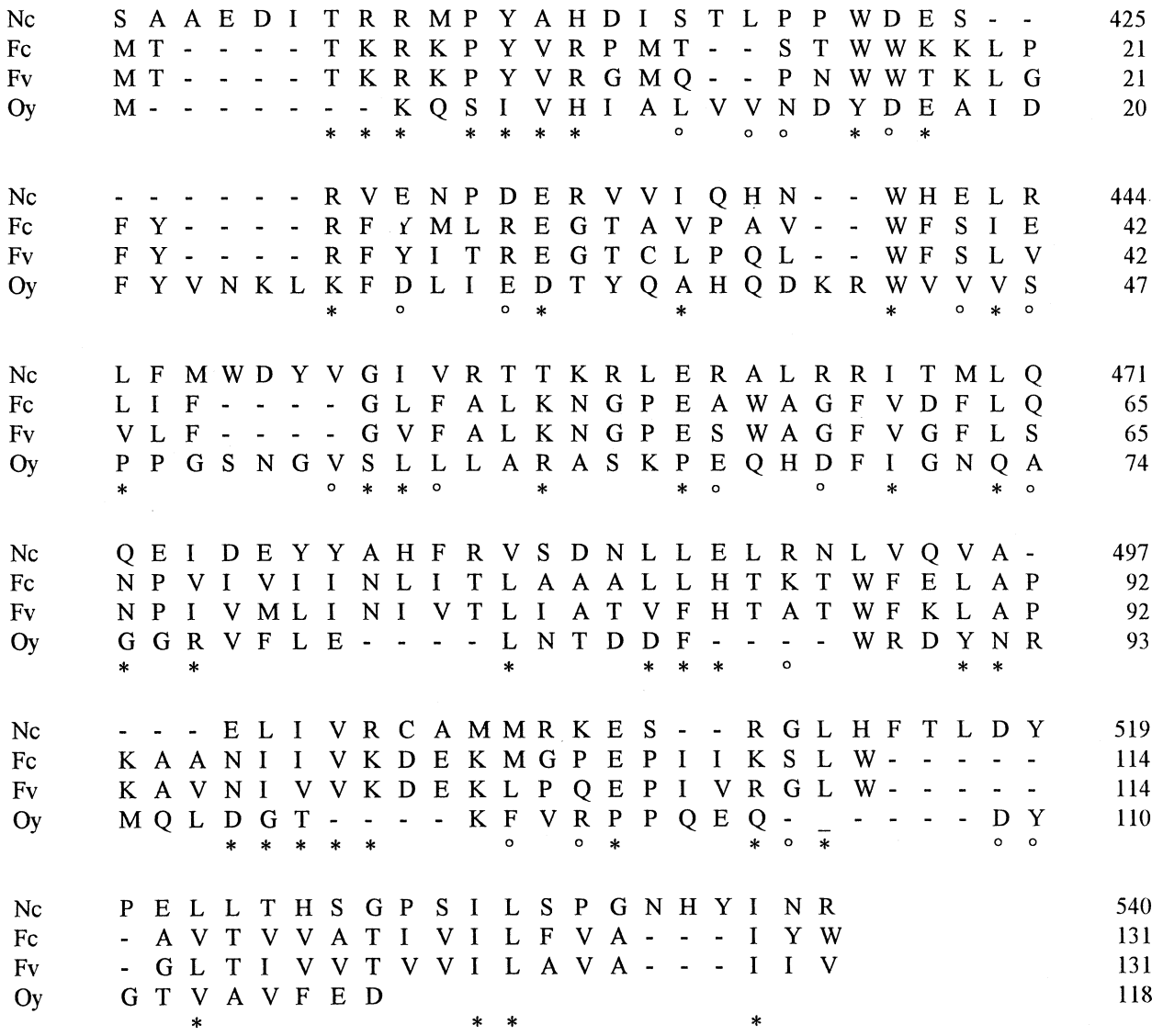


FIGURE 6: Alignment of the amino acid sequence of the C-terminal portion of L-aspartate oxidase from *E. coli* and the FRD-C subunit of fumarate reductases and ORF2 from *Shewanella putrefaciens*. See the text for details and comment. The sequences are labeled as follows: Nc, *E. coli* L-aspartate oxidase (Flachmann et al., 1988) from residue 401; Fc, *E. coli* fumarate reductase C subunit (Westemberg et al., 1993); Fv, *Proteus vulgaris* fumarate reductase C subunit (Cole, 1987); and Oy, ORF2 from *S. putrefaciens* (Pealing et al., 1992). Residues conserved in Nc and in at least two other sequences are indicated below the alignment with an asterisk (*) and are used to calculate the percent similarity; ° indicates the residues conserved in Nc and in one sequence.

Quinone Reductase Activity of L-*Aspartate Oxidase and Sequence Similarity Studies.* Facultative anaerobes such as *E. coli* can grow under either aerobic or anaerobic conditions. They obtain energy from fermentation or from respiration involving the reduction of oxygen or other electron acceptors such as nitrate, nitrite, fumarate, dimethyl sulfoxide, and trimethylamine *N*-oxide. Under anaerobic conditions, quinones are thought to function in the respiratory chain as links between dehydrogenases (such as succinate dehydrogenase) and reductases (such as fumarate reductase) allowing reoxidation of reduced nucleotides and reduction of the final electron acceptor. *E. coli* is capable of synthesizing two quinones, ubiquinone-8 and menaquinone-8, the ratio of which is variable and depends on the growth conditions (Ingledew & Poole, 1984). In particular, utilization of fumarate by fumarate reductase is strictly correlated to the presence of menaquinone or desmethylmenaquinone. The molecule can be reduced by two one-electron steps to menaquinol and is reoxidized by fumarate reductase only in the presence of FRD-C and FRD-D subunits which anchor fumarate reductase to the membrane and are intimately involved in the redox reaction. Analogous membrane-bound fractions were discovered in succinate dehydrogenase which reduces ubiquinone to the corresponding ubiquinol through the formation of a radical species (Nakamura et al., 1996). On the contrary, the data presented in this report clearly indicate that L-aspartate oxidase behaves very differently. The enzyme is reduced only by L-aspartate and shows a lower selectivity for the electron acceptor. Indeed, besides oxygen and fumarate, naphtho- or benzoquinones can be reduced at a comparable rate by the enzyme, in one two-electron step, without the presence of any membrane-bound subunit. Moreover, nitrate, nitrite, and TMNO are not electron acceptors of reduced L-aspartate oxidase under anaerobic conditions. According to Westemberg et al. (1993), reduction or oxidation of quinones by fumarate reductase was proposed to occur at two separate sites, Q_a and Q_b. The spectroscopic and NMR data reported above suggest that a quite different type of structure is operating in L-aspartate oxidase. Only one K_d and one k_{off} could be calculated for

menadione and 2,3-dimethoxy-5-methyl-*p*-benzoquinone, suggesting that the enzyme presents a single quinone binding site where substrate reduction takes place. This domain seems to be quite close to the catalytic site of the molecule as deduced from the marked menadione protection observed in chemical modification experiments. To locate this domain in the L-aspartate oxidase primary structure, sequence similarity studies were carried out with the 15 kDa subunit of fumarate reductase (FRD-C) from different sources. Figure 6 shows the sequence alignment between the C-terminal portion of L-aspartate oxidase and the 15 kDa subunit of fumarate reductase (FRD-C) from *E. coli* and *Proteus vulgaris*. ORF2 corresponds to the protein sequence predicted from a reading frame of 118 codons that extends downstream from the flavocytochrome *c* coding sequence and presents some similarity with the 13 kDa FRD-C (Pealing et al., 1992). Similarity with SDH-D, as well as with FRD-D, is significant but lower than that shown in Figure 6. The results suggest that the quinone binding domain may be located at the last 150 amino acid residues of the molecule which present 31% sequence similarity with FRD-C. This value, although not very high, is quite significant if one considers that the hydrophobic polypeptides associated with different fumarate reductases and succinate dehydrogenases share little sequence similarity among themselves (Ackrell et al., 1991). Remarkably, in FRD-C, few residues are known to be catalytically active. Among these, E30 seems to be essential in quinone oxidation/reduction and H82 is considered to be a partecipant in the Q_b-type site, although its substitution mainly affects quinole oxidation, without altering the fumarate reductase activity (Westemberg et al., 1993). As reported in Figure 6, in L-aspartate oxidase, only E30 residue is conserved, in accordance with the lack of reactivity shown by the enzyme toward reduced quinones. Other observations further support the hypothesis that the C terminus is involved in quinone binding. First of all, this portion of the protein is very rich in hydrophobic residues such as tryptophan and phenylalanine which are very important in quinone binding sites for positioning the molecule for efficient electron transfer. Moreover, secondary structure analyses suggest that the 430−451 portion of the protein may be structured as α-helices which can contribute to the binding by the hydrophobic "face" of the helix (residues I436, W440, L443, M447, and V451). Second, the sequence similarity exists in a region of the flavoprotein primary structure (the C-terminal portion) which shows quite low similarity with FRD-A (Mortarino et al., 1996). Interestingly, all the residues of FRD-C conserved in L-aspartate oxidase are substituted in the flavoprotein subunits of fumarate reductase and succinate dehydrogenase, which are known to be unable to directly interact with quinones. Finally, according to the alignment reported in Mortarino et al. (1996), the relationship between L-aspartate oxidase and the soluble form of fumarate reductase from yeast or flavocytochrome *c* from *Shewanella putrefaciens* does not extend to the last 150 residues. As proposed by Pealing et al. (1992) for ORF2, its absence from the single-subunit, soluble fumarate reductase, flavocytochrome *c*, clearly indicates that it is not required for fumarate reduction *per se*. Keeping with these conclusions, we assume that the C-terminal portion in L-aspartate oxidase is probably involved in quinone binding but it is clearly not necessary for fumarate reduction.

These observations and the kinetic results show that *in vitro* quinones substitute for oxygen in the first step of pyridine nucleotide biosynthesis. This unexpected finding prompted us to further investigate the behavior of L-aspartate oxidase in the presence of lipids in order to understand the possible physiological meaning of the L-aspartate:quinone oxidoreductase activity. Menaquinone and ubiquinone are mainly located in the membranes of *E. coli* cells, and the enzymes able to use these molecules as electron acceptors are reported to be associated with the lipid bilayer. Many of them, however, are considered "peripheral" enzymes and can be readily dissociated from the membrane by salt solutions, sonication, or chelating agents (Cunningham & Hager, 1971). It seems therefore that, even if L-aspartate oxidase is reported to be a cytosolic protein (Nasu et al., 1982), the possibility that the enzyme may interact with quinones in the cellular membrane cannot be ruled out, as suggested by the results obtained using coenzyme Q_7 as the electron acceptor incorporated in the membrane of liposomes.

However, according to Coleman (1973), probably the greatest difference between membrane enzymes and soluble molecules is the interrelationship of many of the former group with lipids. Peripheral proteins such as piruvate oxidase or D-lactate dehydrogenase are sensibly activated by adding phospholipids or liposomes and present strong binding to membrane vesicles. In the case of L-aspartate oxidase, the data reported above show that the enzyme activity using menadione is unaffected by the presence of lipids even after incubation for a long time. Moreover, only a weak binding to *E. coli* membrane vesicles can be detected.

Therefore, on the basis of the results obtained *in vitro*, it is possible to speculate that *in vivo* reduced L-aspartate oxidase under anaerobic conditions may use directly fumarate as the electron acceptor and quinones may not be the electron carriers between the dicarboxylic acid and the enzyme. However, the possibility that quinones may be used in the absence of fumarate or oxygen cannot be ruled out. Further studies on this subject are necessary in order to confirm such a hypothesis.

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