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Influence of Vesicle Size and Oxidase Content on Respiratory Control in Reconstituted Cytochrome Oxidase Vesicles†

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ABSTRACT: Previous work has shown that the respiratory control or coupling exhibited by reconstituted cytochrome oxidase systems can be markedly sensitive to the lipid composition, the lipid to protein ratio, and the vesicle size. In this work we have attempted to ascertain which, if any, of these factors plays a definitive role in determining the observed coupling. Vesicles prepared from dioleoylphosphatidylcholine–dioleoylphosphatidylethanolamine (1:4) were fractionated by DEAE chromatography to obtain a population homogeneous with respect to their lipid to protein ratio. This subpopulation was then applied to a Sepharose 4B-CL column to separate the vesicles by size. Fractions eluted from the column were assayed for enzyme activity in the presence and absence of carbonyl cyanide (trifluoromethoxy)phenylhydrazone plus valinomycin. The coupling ratio was found to be dependent upon vesicle size; the smaller the vesicles, the higher the ratio. This suggests that lipid composition per se does not determine coupling characteristics. Reducing vesicle size for a given lipid to protein ratio has two effects. First, the radius of curvature is increased and, second, the average number of oxidase molecules per vesicle is reduced. In order to identify which of these factors was responsible for the observed tighter coupling, the oxidase was reconstituted with dioleoylphosphatidylcholine into vesicles of a defined size and the number of oxidase molecules per vesicle varied. The highest coupling ratios were observed for vesicles containing on average only one oxidase dimer. As the fraction of vesicles containing more than one protein was increased, the coupling ratio rapidly declined. Mathematical analysis of these results is consistent with the proposal that in reconstituted oxidase vesicles containing two or more oxidase dimers, oxidase complexes with an inward orientation (such that they cannot interact with external cytochrome *c*) can uncouple outwardly oriented oxidase. Further, it was observed that vesicles containing a single oxidase dimer could be resolved by DEAE chromatography into subpopulations where the protein was oriented either facing outward or facing the interior of the vesicle.

Cytochrome *c* oxidase catalyzes the reduction of molecular oxygen to water, which is the terminal reaction in the mitochondrial electron transport chain. It is one of the best characterized integral membrane proteins and has been shown to be a Y-shaped multisubunit enzyme where the two arms of the Y span the inner mitochondrial membrane and the stalk (which contains the cytochrome *c* binding site) extends from the cytoplasmic side (Deatherage et al., 1982). It can be isolated and purified following detergent solubilization and can be reconstituted into vesicles with a well-defined lipid composition. It has been demonstrated that optimal activity is obtained when the enzyme is reconstituted with phospholipids that contain long unsaturated acyl groups (Vik & Capaldi, 1977).

Cytochrome oxidase serves to generate and maintain a transmembrane proton gradient in vivo, and it is therefore vital that the enzyme be well sealed in the membrane so that such gradients are not immediately dissipated. A measure of such sealing is provided by the respiratory control or coupling, given by the ratio of the enzyme activity in the presence of uncoupling agents (a proton ionophore such as FCCP[1] and the K^+ ionophore valinomycin) to that in the absence of uncouplers. Reconstituted oxidase vesicles exhibiting good respiratory control ratios can be generated (Hunter & Capaldi, 1976; Racker, 1973); however, the coupling ratios obtained are sensitive to a variety of factors. Vik & Capaldi (1977) have observed that well-coupled systems are only obtained at high lipid to protein ratios whereas other workers have shown that coupling is dependent on the lipid composition itself (Racker, 1973). Alternatively, it has been suggested that the size of the vesicle containing the oxidase may also influence respiratory control (Madden et al., 1983).

In this work we have approached this somewhat confused situation with the aim of determining whether lipid composition, protein content, or vesicle size plays a definitive role in determining the coupling ratios exhibited by reconstituted oxidase systems. We show that neither the lipid composition nor the vesicle size plays a primary role but that the oxidase content per vesicle has a marked influence. In particular, optimum coupling is obtained in vesicles containing one oxidase dimer per vesicle, and the coupling ratios decrease dramatically as the protein content is raised. These results are analyzed in terms of a model whereby in reconstituted vesicles con-

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[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; SDS, sodium dodecyl sulfate; FCCP, carbonyl cyanide (trifluoromethoxy)phenylhydrazone; OGP, octyl β-D-glucopyranoside.

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taining two or more oxidase dimers the presence of an inwardly oriented oxidase (which has the cytochrome *c* binding site on the interior side of the vesicle) could act as an ion channel to uncouple outwardly oriented oxidase.

Materials and Methods

Cytochrome *c* oxidase was prepared by the method of Kuboyama et al. (1972). The oxidase was finally resuspended in 1% Tween-80 and stored in liquid nitrogen at a protein concentration of approximately 50 mg/mL. A molecular weight of 260000 was assumed for the oxidase dimer (Ozawa et al., 1982). DOPC and DOPE were synthesized as described previously (Cullis & De Kruijiff, 1976) and were at least 99% pure as determined by thin-layer chromatography. Soybean phospholipids (Associated Concentrates) were purified as described by Kagawa & Racker (1971).

Cytochrome *c* (horse heart, type VI), sodium cholate, Tween-80, carbonyl cyanide (trifluoromethoxy)phenylhydrazone (FCCP), and valinomycin were obtained from Sigma. Ascorbic acid was purchased from Matheson Coleman and Bell whereas octyl β-D-glucopyranoside was obtained from Calbiochem.

For reconstitution experiments 15 mg of lipid and 7.5 mg of cholate were cosonicated in 1 mL of 50 mM potassium phosphate, pH 8.3, on ice for 3 min in a Fisher Model 150 sonicator at 65% of maximum power output. Cytochrome *c* oxidase (1 mg, 100 or 50 μg) was then added and the mixture dialyzed against 300 volumes of 50 mM potassium phosphate, pH 8.3, for 5 h and then against 500 volumes of the same buffer for an additional 10 h. The mixture was then applied to a DEAE-Sephacel column (0.9 × 15 cm) preequilibrated with 10 mM potassium phosphate at pH 8.3. The reconstituted vesicles were eluted with a potassium phosphate gradient as indicated under Results at a flow rate of 5 mL h^{-1}.

Vesicles prepared from cytochrome oxidase and DOPC–DOPE (1:4) of lipid to protein ratio 860:1 were sized on a Sepharose 4B-CL column (1.5 × 30 cm). They were eluted in 100 mM potassium phosphate, pH 8.3, at a flow rate of 3 mL h^{-1} at 4 °C.

Enzyme assays were carried out as described previously (Madden et al., 1983). Lipids were extracted by the method of Folch et al. (1957) and quantified by determination of inorganic phosphate as described by Chen et al. (1956). Protein concentrations were quantified either by the method of Lowry et al. (1951) or in the case of vesicles with high lipid to protein ratios by determination of the heme aa_3 concentration from the reduced minus oxidized spectrum, 605–630 nm. The vesicles were first solubilized in 1% Tween-80 and an extinction coefficient of 27 mM^{-1} assumed for the aa_3 unit (Nicholls et al., 1980).

Samples for freeze–fracture were prepared as described previously (Madden et al., 1983). They were fractured and visualized employing a Balzers freeze–fracture apparatus and a Phillips 400 electron microscope.

The orientation of the reconstituted oxidase was determined by the spectroscopic procedure of Nicholls et al. (1980). Difference spectra were obtained at 25 °C on a Pye Unicam SP8-500 spectrophotometer.

Results

As indicated in the introduction the respiratory control exhibited by reconstituted cytochrome oxidase systems can be highly sensitive to the lipid species employed for reconstitution. In mixtures of DOPC and DOPE, for example, maximal coupling was observed for DOPC:DOPE (1:4) ratios whereas little or no coupling was observed when the enzyme was reconstituted with DOPC or DOPE alone (Madden et al., 1983). It was further observed that the well-coupled reconstituted systems were smaller than their uncoupled counterparts.

Table I: Effect of Vesicle Size on Enzyme Coupling

elution volume on 4B-CL (mL)	lipid to protein molar ratio	av vesicle diameter (nm)	coupling ratio
14	790:1	64	1.01
17	830:1	58	1.44
19	880:1	52	1.68
36	890:1	31	4.23
41	880:1	26	4.44

In order to ascertain more precisely the source of the variability in coupling, a reconstituted oxidase system with defined lipid to protein content was prepared (employing the DOPC–DOPE, 1:4, mixture), which could subsequently be subfractionated according to vesicle size. Any observed variability in respiratory control between these well-defined subfractions can then be related more directly to the influence of the size of the reconstituted system. As we have indicated elsewhere (Madden et al., 1983), obtaining a reconstituted oxidase preparation with a defined lipid to protein content is not necessarily trivial, as the standard reconstitution protocol results in a heterogeneous system in which many vesicles are devoid of protein and others contain varying amounts of the enzyme. This has also been observed by other workers (Fuller et al., 1982; Carroll & Racker, 1977). The subpopulations with varying oxidase content can, however, be conveniently separated by DEAE chromatography as shown in Figure 1a where the elution profile of cytochrome oxidase reconstituted with DOPC–DOPE (1:4) is illustrated. The vesicles eluted in peak 1 contain no protein, while peaks 2–4 contain vesicles with lipid to protein ratios of 1800:1, 860:1, and 720:1, respectively, which exhibit corresponding respiratory control ratios of 7.28, 3.24, and 2.62. The vesicle size distributions in the protein-containing fractions were determined employing freeze–fracture electron microscopy (van Venetië et al., 1980) and are shown in Figure 1b. It may be observed that the vesicles with the highest lipid to protein ratios (which exhibit the best coupling) are also the smallest. The vesicles from peak 3 (lipid to protein ratio 860:1) were then subfractionated according to size employing a Sepharose 4B-CL column, giving rise to the elution profile of Figure 2. The vesicle size and respiratory control ratio were determined at the points in the elution profile indicated by the arrows, and the relation between the coupling and vesicle diameter is given in Table I. It may be noted that among these subpopulations, which exhibited similar lipid to protein ratios (within 10%), the smallest vesicles exhibited much higher respiratory control.

These results clearly establish that for a lipid to protein ratio of 860:1 (mol:mol) the smallest reconstituted oxidase systems are the most tightly coupled. Two possible explanations of this behavior are that the reduced amounts of protein per vesicle in the smaller systems somehow contribute to vesicle impermeability or that the smaller vesicle size per se leads to improved sealing characteristics. It is clear that the particular lipid mixture (DOPC–DOPE, 1:4) employed does not play a decisive role in determining coupling properties, as all these subpopulations exhibited identical lipid compositions. Thus it was decided to reconstitute oxidase at higher lipid to protein ratios (thus reducing the amount of enzyme incorporated per vesicle) into pure DOPC systems, which gives rise to somewhat larger vesicles than the DOPC–DOPE (1:4) mixture on detergent dialysis. The oxidase was therefore reconstituted with DOPC at lipid to protein molar ratios of 5000:1, 50000:1, and

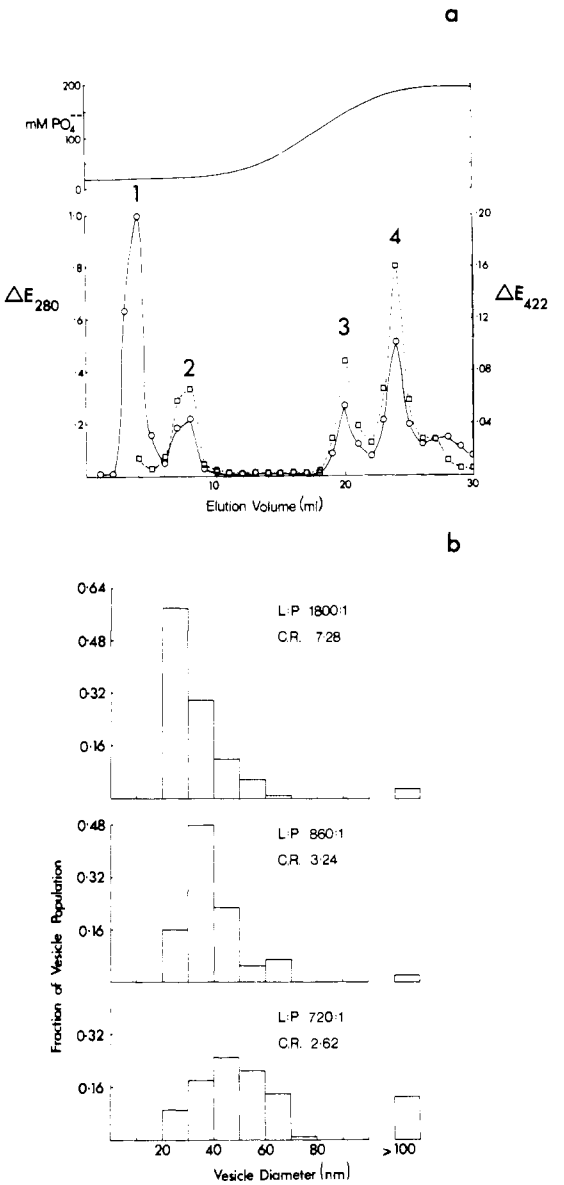

FIGURE 1: (a) Elution profile of cytochrome oxidase reconstituted with DOPC–DOPE (1:4) on DEAE-Sephacel. The molar ratio of lipid to protein used was 5000:1. Absorbance at 280 nm (○); absorbance at 422 nm (□). (b) Size distribution of vesicles eluted in peaks 2–4 indicating the lipid to protein ratio and coupling ratios.

100 000:1, and elution profiles with four peaks, similar to the elution profile of Figure 1a, were obtained on DEAE-Sephacel chromatography of these reconstituted systems. As indicated in Table II, the coupling obtained at various points in the elution profile for the lower lipid to protein ratio (5000:1) is relatively poor, consistent with previous results (Madden et al., 1983). However, oxidase–DOPC preparations with higher lipid to protein ratios can exhibit excellent coupling. This is particularly true of the oxidase-containing systems obtained in peak 2 of the elution profile, which are eluted immediately after the void volume, for which a coupling ratio of 8 is obtained in the DOPC–oxidase (100 000:1) system. This improved coupling cannot be attributed to smaller reconstituted systems as the size of the well-coupled vesicles (determined by freeze–fracture electron microscopy) is similar to that of oxidase–DOPC vesicles exhibiting poor respiratory control (see Table II).

The results to this stage show that neither the vesicle size nor the lipid composition is a determining factor in obtaining well-coupled reconstituted oxidase systems. The oxidase

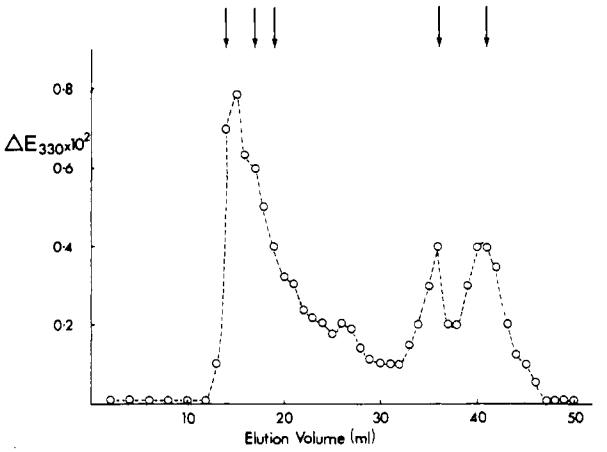

FIGURE 2: Elution profile on Sepharose 4B-CL of vesicles from peak 3 on the DEAE-Sephacel column. The oxidase was reconstituted with DOPC–DOPE (1:4) and fractionated as shown in Figure 1. The lipid to protein molar ratio of the vesicles applied to the Sepharose 4B-CL column was 860:1. The arrows indicate the fractions that were assayed for enzyme coupling ratio and vesicle size (results shown in Table I).

Table II: Coupling Ratio as a Function of the Average Number of Oxidase Dimers per Vesicle

initial molar ratio of lipid to protein	peak no. on DEAE column	av vesicle diameter (nm)	av no. of dimers per vesicle	coupling ratio
5 000:1	2	62	2.13	2.23
5 000:1	3	69	2.29	2.02
5 000:1	4	71	4.63	1.28
50 000:1	2	51	0.98	7.39
50 000:1	3	56	1.15	5.22
50 000:1	4	64	1.22	4.97
100 000:1	2	61	1.01	8.00
100 000:1	3	74	1.03	6.07
100 000:1	4	69	1.18	4.70

content itself appears to have a marked influence, however, supporting the possibility that the number of oxidase molecules per vesicle is the important variable affecting control ratios. We therefore analyzed the oxidase content of the DOPC–oxidase systems employing freeze–fracture procedures. As illustrated in Figure 3, the enzyme can be clearly visualized as an intramembrane particle, and for preparations containing relatively high amounts of protein [e.g., elution peak 4 of the DOPC–oxidase (5000:1) system] many particles are present per vesicle as observed in Figure 3a. Alternatively, in preparations containing low amounts of protein [e.g., elution peak 2 of the DOPC–oxidase (50 000:1) system] much fewer particles are visible (Figure 3c). By counting the number of particles associated with equatorially shadowed vesicles [i.e., vesicles fractured exactly in half; see van Venetië et al. (1980)] and multiplying by a factor of 4 to account for the fact that freeze–fracture particles can only be visualized on one-quarter of the surface of any given vesicle, one can estimate the number of particles per vesicle. As indicated in Table II, the vesicles exhibiting the higher coupling ratios contain one particle per vesicle, and the coupling decreases very sharply as the protein content is increased.

The relation between the intramembranous particles observed in oxidase-containing vesicles and the oxidase molecule itself is clearly important for analysis of these results. We therefore determined the number of oxidase monomers present per vesicle in the preparations exhibiting one particle per vesicle. It was found that the particles represent dimers of cytochrome oxidase, in agreement with previous results

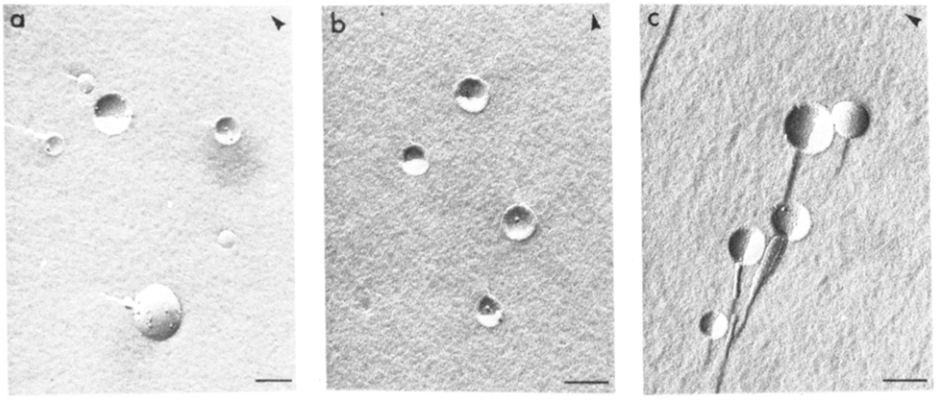

FIGURE 3: Freeze–fracture electron micrographs of reconstituted cytochrome oxidase vesicles: (a) vesicles from peak 4 of the DEAE-Sephacel elution profile (lipid to protein ratio employed for reconstitution = 5000:1); (b) vesicles from peak 2 of the DEAE-Sephacel elution profile (lipid to protein ratio employed for reconstitution = 5000:1); (c) vesicles from peak 2 of the DEAE-Sephacel elution profile (lipid to protein ratio employed for reconstitution = 50 000:1). The arrowheads indicate the direction of shadowing and the bars represent 100 nm in each micrograph.

(Deatherage et al., 1982). This observation, in combination with the results of Table II, clearly indicates that the most tightly coupled reconstituted oxidase systems are those containing one oxidase dimer per vesicle.

The ability of DEAE column chromatography to resolve vesicles containing one oxidase dimer from other components is rather remarkable and leads to questions concerning the transmembrane orientation of the oxidase molecules in these systems. In particular, in its native configuration the polar region of the oxidase complex on the cytoplasmic side of the inner mitochondrial membrane is considerably larger than the polar region on the matrix side. It is therefore likely that the ability of DEAE chromatography to resolve between reconstituted oxidase vesicles with different protein contents will depend primarily on the amount of oxidase molecules oriented with the larger polar region on the exterior of the vesicle. In turn, this would suggest that the well-coupled systems containing only one oxidase per vesicle that are retarded on the DEAE column might have an asymmetric orientation similar to that observed in the inner mitochondrial membrane. The orientation of oxidase in the DOPC–oxidase (50 000:1) reconstitute was therefore examined by employing the spectroscopic procedure of Nicholls et al. (1980). As indicated in Table III, prior to DEAE chromatography the oxidase is nearly randomly incorporated with 54% of the heme-containing moieties available on the vesicle exterior and 46% sequestered. After DEAE chromatography (elution peak 2), however, a marked asymmetry is observed with 92% of the heme groups oriented outward and only 8% apparently sequestered in the vesicle interior. Thus the single oxidase dimer contained in these vesicles has a largely uniform transmembrane orientation.

These observations have two consequences. First, it would be expected that the specific activity of the oxidase containing vesicles (in the presence of FCCP and valinomycin) should be significantly higher in the peak 2 vesicles than in the unfractionated systems. This is because nearly all the oxidase contained in the peak 2 fraction will be available to cytochrome *c*, whereas only 54% will be available in the mixture prior to DEAE chromatography. As indicated in Table III, the specific activity is indeed nearly a factor of 2 higher in the peak 2 fractions than in the unfractionated systems.

A second point is that if the resolving power of the DEAE column relies on the presence of outwardly oriented oxidase, then vesicles containing only inwardly oriented oxidase should not be retained and should be eluted in the void volume. It was found for the 50 000:1 and 100 000:1 DOPC–oxidase systems that significant amounts of protein were indeed contained in the void volume, but no oxidase activity could be observed on addition of cytochrome *c*. However, the subsequent addition of the detergent octyl β-D-glucopyranoside to the assay medium revealed latent oxidase activity. This is fully consistent with the proposal that vesicles containing only inwardly oriented oxidase are not retarded on the column and that the associated enzyme activity can only be expressed when the vesicles are disrupted by detergent, resulting in exposure of the enzyme to external cytochrome *c*. It may be noted that the addition of similar levels of detergent to the peak 2 subfraction did not result in any enhancement of oxidase activity.

Table III: Specific Activity and Orientation of Reconstituted Cytochrome Oxidase

sample	specific activity [μmol of ferrocytochrome *c* oxidized min^{-1} (nmol of heme *a*)$^{-1}$]	orientation of oxidase: % facing out	orientation of oxidase: % facing in
mixture[a] prior to separation on DEAE column	7.82	54	46
vesicles from peak 2 on DEAE column	13.94	92	8

[a] DOPC–oxidase vesicles were prepared at an initial molar ratio of lipid to protein of 50 000:1 as described under Materials and Methods. The profile of the vesicles on the DEAE column is similar to that shown in Figure 1a.

Discussion

This work provides new and definitive information regarding the influence of lipid composition, vesicle size, and protein content on the coupling ratios exhibited by reconstituted oxidase systems, which also has interesting implications for certain properties of the oxidase molecule itself. Further, some of the reconstituted systems characterized here have novel properties that may well be of general utility. We discuss these two areas in turn.

The results presented here clearly show that for oxidase reconstituted with DOPC–DOPE (1:4) or pure DOPC, the details of the lipid composition or vesicle size are not determining factors for obtaining well-coupled systems. The protein content, on the other hand, has a marked influence as optimum coupling is obtained when a single oxidase dimer is present per vesicle and such coupling rapidly declines as the protein content is increased. This observation explains the strong

dependence of the coupling ratio on the lipid composition in DOPC–DOPE–oxidase systems with relatively low lipid to protein ratios, as the size of the reconstituted vesicle is determined by the lipid composition. As indicated previously (Madden et al., 1983), increasing the DOPE content in DOPE–DOPC–oxidase systems results in production of progressively smaller reconstituted vesicles. The higher coupling observed for DOPC–DOPE (1:4) vesicles may therefore be attributed to their small size, which, for a fixed lipid to protein ratio, leads to a reduced number of oxidase molecules per vesicle. This will result in superior coupling. Similarly, it has been noted (Hinkle et al., 1972) that reconstitution of oxidase with asolectin (a partially purified preparation of soybean phospholipids) results in well-coupled systems. This lipid mixture results in production of very small vesicles on detergent dialysis [average diameter 27 nm (T. D. Madden, unpublished experiments)] and will therefore contain fewer proteins per vesicle when reconstituted with oxidase. It may also be noted that for such small vesicles the oxidase is incorporated with a preferred orientation such that a majority of the enzyme faces the exterior. This is likely due either to steric restrictions or to the high radius of curvature of such vesicles and, in the light of the following discussion, will also lead to improved coupling ratios.

It is of interest to ascertain why a reconstituted oxidase–lipid system should be tightly coupled when each vesicle contains a single oxidase dimer and why the coupling ratios should decrease rapidly as the protein content per vesicle is increased. It may be suggested that protein–protein interactions in the systems containing two or more oxidase molecules somehow generate defects in the membrane through which ions can pass. It is difficult to eliminate such a possibility save to note that there is little evidence from freeze–fracture studies for any clustering of oxidase molecules in the reconstituted systems. A more attractive proposal is prompted by the studies of Wikstrom et al. (1981), who suggest that the oxidase dimer contains a proton channel. If this is the case, then an oxidase dimer oriented inwardly could provide an ion channel that could dissipate any gradient generated by outwardly oriented oxidase. It is instructive to ascertain whether such a proposal would explain the very rapid decrease in coupling ratios observed as the protein content of reconstituted oxidase systems increases. We assume that, in a vesicle containing n proteins, if one or more proteins have an inward orientation (are not able to interact with external cytochrome c), then any outwardly oriented oxidase dimers are uncoupled. A straightforward analysis for the general case where the probability of a protein exhibiting an outward orientation (P_o) is different than the probability of an inward orientation (P_i) reveals that for a protein to vesicle ratio x the coupling ratio is given by

$$R(x) = R(1)\left[\sum_{m=1}^{n} P(n,m) + C(x,n)\sum_{m=1}^{n+1} P(n+1,m)\right] / \left\{P_o{}^n n + C(x,n)P_o{}^{n+1}(n+1) + R(1)\left[\sum_{m=1}^{n-1} P(n,m) + C(x,n)\sum_{m=1}^{n} P(n+1,m)\right]\right\} \quad (1)$$

where $P(n,m) = [n!/m!(n-m)!]P_i{}^{n-m}P_o{}^m m$, $C(x,n) = x - n/(n+1-x)$, $n \le x \le n+1$, and $R(1)$ is the coupling ratio achieved for one protein per vesicle. For the simple case where $x = n$ and $P_i = P_o = 0.5$, eq 1 reduces to

$$R(n) = \frac{2^{n-1}R(1)}{1 + (2^{n-1} - 1)R(1)} \quad (2)$$

As illustrated in Figure 4 these equations predict a very rapid decrease in the coupling ratio R as the oxidase content is increased, which correlates remarkably well with the observed decrease in R for increased protein to vesicle ratios. This supports the interpretation that in reconstituted vesicles containing two or more oxidase dimers the presence of inwardly oriented oxidase uncouples remaining outwardly oriented enzyme. While this would be consistent with the oxidase containing an ion channel, it should be mentioned that in the reconstitution procedure employed the initial level of cholate is not sufficient to completely solubilize the phospholipid. Thus, it is likely that during dialysis the oxidase is incorporated into preformed vesicles. This might require the large polar cytochrome c binding region of the complex to pass through the hydrophobic membrane interior, which could conceivably cause a structural perturbation of the enzyme leading to the formation of a "channel".

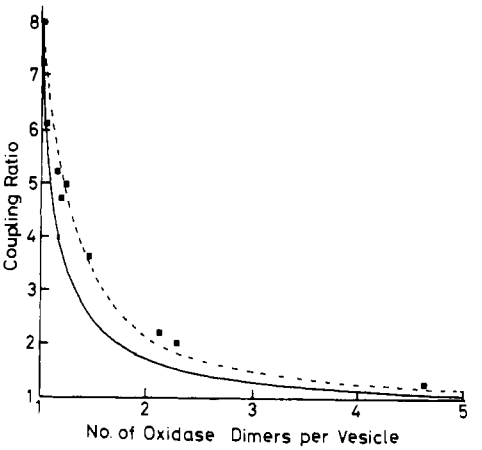

FIGURE 4: Theoretical coupling ratios R as a function of the protein to vesicle ratio as calculated from eq 1 (see the text) assuming $R(1) = 8.0$. The solid line represents the coupling ratios assuming an equal probability of outwardly oriented oxidase (P_o) and inwardly oriented oxidase (P_i), whereas the dashed line was obtained assuming $P_o = 0.6$ and $P_i = 0.4$. The experimental coupling ratios observed for various protein contents are given by (■).

There are two aspects of the reconstituted oxidase systems generated in this work that merit further emphasis. First, reconstitution at high lipid to protein ratios, in combination with DEAE column chromatography, can result in a relatively homogeneous preparation containing one protein dimer per vesicle. Such a system could be of utility in characterizing phenomena as due to protein–protein interactions or due to an intrinsic property of the protein itself. Second, these reconstituted systems can exhibit asymmetric transbilayer distributions of incorporated protein such that the large majority (>90%) of the oxidase can interact with external cytochrome c or alternatively where the cytochrome c binding site is sequestered in the vesicle interior. These are clearly most attractive model systems, potentially allowing the influence on protein function of variables acting on one side or the other of the membrane in vivo to be investigated in a well-defined manner.

In summary, the results presented here demonstrate that in reconstituted oxidase vesicles the presence of only one protein per vesicle results in optimal coupling, which rapidly decreases as the protein content is increased. This observation is consistent with an ability of inwardly oriented oxidase to provide an ion channel to dissipate chemical gradients generated by oxidase able to interact with external cytochrome c. Finally, techniques that allow the preparation of reconstituted vesicles that contain a single oxidase dimer per vesicle and where the dimer exhibits a well-defined transbilayer orientation are demonstrated.

Acknowledgments

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Registry No. DOPC, 10015-85-7; DOPE, 2462-63-7; cytochrome oxidase, 9001-16-5.

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One-Electron Oxidation–Reduction Properties of Hepatic NADH–Cytochrome b_5 Reductase†

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ABSTRACT: The one-electron oxidation–reduction properties of flavin in hepatic NADH–cytochrome b_5 reductase were investigated by optical absorption spectroscopy, electron paramagnetic resonance (EPR), and potentiometric titration. An intermediate with a peak at 375 nm previously described by Iyanagi (1977) [Iyanagi, T. (1977) *Biochemistry 16*, 2725–2730] was confirmed to be a red anionic semiquinone. The NAD^+-bound reduced enzyme was oxidized by cytochrome b_5 via the semiquinone intermediate. This indicates that electron transfer from flavin to cytochrome b_5 proceeds in two successive one-electron steps. Autoxidation of the NAD^+-bound reduced enzyme was slower than that of the NAD^+-free reduced enzyme and was accompanied by the appearance of an EPR signal. Midpoint redox potentials of the consecutive one-electron-transfer steps in the presence of excess NAD^+ were $E_{m,1} = -88$ mV and $E_{m,2} = -147$ mV at pH 7.0. This corresponds to a semiquinone formation constant of 8. The values of $E_{m,1}$ and $E_{m,2}$ were also studied as a function of pH. A mechanism for electron transfer from NADH to cytochrome b_5 is discussed on the basis of the one-electron redox potentials of the enzyme and is compared with the electron-transfer mechanism of NADPH–cytochrome P-450 reductase.

NADH–cytochrome b_5 reductase (EC 1.6.2.2), a single-subunit, mono-FAD-containing enzyme, and cytochrome b_5 function as an electron-transport chain from NADH to a terminal oxidase desaturase in the endoplasmic reticulum (Strittmatter et al., 1974; Ohnishi et al., 1975; Okayasu et al., 1981). The flavoprotein accepts two electrons from NADH with concomitant production of a long-wavelength-absorbing NAD^+-reduced flavoprotein charge-transfer complex and can transfer reducing equivalents from NADH to the one-electron acceptor cytochrome b_5 (Strittmatter, 1965).

Strittmatter (1965) has proposed that by using both the fully reduced flavin and the semiquinone as reductants, NADH–cytochrome b_5 reductase can reduce 2 equiv of cytochrome b_5 in successive one-electron steps. In a previous study (Iyanagi, 1977), we demonstrated that addition of NAD^+ to the partially reduced enzyme yields a new intermediate with an absorption band at 375 nm, showing the characteristic features of a red semiquinone. Hemmerich & Massey (1979) have suggested that in a flavoprotein involved in one-electron transfers, the blue neutral semiquinone is an obligatory intermediate. Indeed, a blue semiquinone is observed in NADPH–cytochrome P-450 reductase (Masters et al., 1965; Iyanagi & Mason, 1973; Yasukochi et al., 1979; Oprian & Coon, 1982) and NADPH–adrenodoxin reductase (Lambeth & Kamin, 1977; Kitagawa et al., 1982). On the other hand, enzymes of the dehydrogenase–oxidase group generate a red-colored radical species. Thus, the observed anionic red semiquinone in the dehydrogenase–electron transferase NADH–cytochrome b_5 reductase is rather unusual. A more

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