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Bovine Heart NADH−Ubiquinone Oxidoreductase Contains One Molecule of Ubiquinone with Ten Isoprene Units as One of the Cofactors†

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ABSTRACT: NADH−ubiquinone oxidoreductase (Complex I) is located at the entrance of the mitochondrial electron transfer chain and transfers electrons from NADH to ubiquinone with 10 isoprene units (Q_{10}) coupled with proton pumping. The composition of Complex I, the largest and most complex proton pump in the mitochondrial electron transfer system, especially the contents of Q_{10} and phospholipids, has not been well established. An improved purification method including solubilization of mitochondrial membrane with deoxycholate followed by sucrose gradient centrifugation and anion-exchange column chromatography provided reproducibly a heme-free preparation containing 1 Q_{10}, 70 phosphorus atoms of phospholipids, 1 zinc ion, 1 FMN, 30 inorganic sulfur ions, and 30 iron atoms as the intrinsic constituents. The rotenone-sensitive enzymatic activity of the Complex I preparation was comparable to that of Complex I in the mitochondrial membrane. It has been proposed that Complex I has two Q_{10} binding sites, one involved in the proton pump and the other functioning as a converter between one and two electron transfer pathways [Ohnishi, T., Johnson, J. J. E., Yano, T., LoBrutto, R., and Widger, R. W. (2005) *FEBS Lett*. *579*, 500−506]. The existence of one molecule of Q_{10} in the fully oxidized Complex I suggests that the affinity of Q_{10} to one of the two Q_{10} sites is greatly dependent on the oxidation state and/or the membrane potential and that the Q_{10} in the present preparation functions as the converter of the electron transfer pathways which should be present in any oxidation state.

It has been proposed that NADH−ubiquinone oxidoreductase (Complex I),[1] the largest proton pump in the electron transfer chain, contains 45 different subunits, 1 FMN, and 8 iron−sulfur clusters, giving an L-shaped overall structure in the electron microscopic image (*1*−*5*). Recently, the X-ray structure of the hydrophilic arm of a bacterial Complex I protruding to the cytoplasmic space has been determined at 3.1 Å resolution, which contains FMN and the iron−sulfur clusters (*6*, *7*). The other arm is likely to be embedded in the membrane to reduce ubiquinone (*8*).

Although many purification protocols have been reported (*9*−*15*) since the discovery of this enzyme 48 years ago by Hatefi et al. (*16*), the composition of the enzyme has not been completely established. Especially the content of the intrinsic Q_{10} is still seriously controversial. Purified bovine Complex I preparations reported thus far contain Q_{10} in far lower than the stoichiometric amount (0.3 or so), suggesting that Q_{10} interacts with Complex I simply as one of the substrates. On the other hand, it has been reported that Complex I from *Escherichia coli* contains a stoichiometric amount of ubiquinone (*17*). Furthermore, two distinct protein-bound ubisemiquinone species, fast relaxing SQ_{Ns} and slow relaxing SQ_{Nf}, have been shown by EPR analyses (*5*, *18*). It was proposed that SQ_{Nf} is involved in the proton pumping, and SQ_{Ns} functions as a converter between one and two electron transfer pathways in Complex I. These results strongly suggest the existence of Q_{10} as the intrinsic constituents or cofactors in Complex I (*5*, *18*). The apparent inconsistency could be due to low consistency in the integrity between the preparations isolated from different procedures. Careful quantifications for iron, sulfur, and FMN which are prerequisite for structural and functional studies on this enzyme have not been accomplished.

Here we report an improved purification procedure for bovine heart Complex I giving a heme-free preparation containing 1 Q_{10}, 1 zinc ion, 70 phosphorus atoms, 30 iron atoms, and 30 inorganic sulfurs (S^{2-}) as the intrinsic constituents. The enzyme preparation shows that Q_{10} is indispensable for the rotenone-sensitive enzyme activity comparable to that determined by using the mitochondrial membrane.

MATERIALS AND METHODS

Experimental procedures for purification and chemical analyses were highly improved for bovine heart Complex I, as given below.

Isolation of the Mitochondrial Membrane Fraction. Bovine heart muscle of one fresh bovine heart after careful removal of fat and connective tissues was minced to prepare 1000 g of minced meat, and a 500 g portion was suspended in 3250 mL of 23 mM sodium phosphate buffer, pH 7.4, at 0 °C and homogenized for 5 min at 13000 rpm in a homogenizer (Nihon Seiki), followed by centrifugation for 20 min at 2800 rpm in a

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[1]Abbreviations: Q_{10}, ubiquinone with 10 isoprene units; Complex I, NADH−ubiquinone oxidoreductase.

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large-scale refrigerated centrifuge (Kubota Model 9810) using an RS-6600 rotor. The other 500 g portion was also treated with the same procedure. The combined precipitate was suspended in 3375 mL of 22.2 mM sodium phosphate buffer, pH 7.4, and rehomogenized, followed by centrifugation with the same procedure as before. All of the supernatants were combined and centrifuged for 30 min at 10000 rpm with a refrigerated centrifuge, Beckman Model Avanti HP-30I using a JLA-10.500 rotor. The precipitate, suspended in 50 mM Tris-HCl buffer, pH 8.0, was centrifuged for 30 min at 30000 rpm with an ultracentrifuge, Beckman Model-7, using a 45 Ti rotor. The precipitate was suspended in 50 mM Tris-HCl buffer, pH 8.0, containing 660 mM sucrose, adjusting the protein concentration at 23 mg/mL. The protein concentration was determined by the Markwell procedure(*19*).

Purification of Bovine Heart Complex I. The mitochondrial membrane fraction at 23 mg of protein/mL in 50 mM Tris-HCl buffer, pH 8.0, containing 0.66 M sucrose, isolated from 1000 g of bovine heart muscle as described above, was treated with 0.73% (w/v) sodium deoxycholate (Dojindo, the highest grade) in the presence of 7.2% (w/v) KCl at 0 °C with stirring for 15 min. The supernatant obtained by centrifugation at 35000 rpm for 30 min was diluted with cold deionized water of $^1/_{14}$th volume of the supernatant, followed by centrifugation at 30000 rpm at 30 min. The resulting supernatant was centrifugated under a stepwise sucrose gradient (2.0, 1.1, 1.0, and 0.9 sucrose in 50 mM Tris-HCl, pH 8.0, and 0.2% dodecyl maltoside) at 105000*g* for 15 h. The enzyme fraction identified by the enzyme activity was further purified by anion-exchange column chromatography using Poros-20HQ (Applied Biosystems) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 0.2% dodecyl maltoside and 660 mM sucrose. The enzyme fractionation was developed with linear increase in KCl concentration up to 0.4 M. The enzyme fraction eluated around 0.35 M KCl was precipitated by ammonium sulfate (350 g/L). The precipitate, dissolved in a minimal volume of 50 mM Tris-HCl buffer, pH 8.0, containing 0.2% dodecyl maltoside and 660 mM sucrose, was dialyzed against 40 mM Hepes-Na buffer, pH 7.8, containing 10% (v/v) glycerol.

Enzyme Activity Assay Procedure. The reaction mixture of 2.12 mL contained 150 μM NADH and 50 μM ubiquinone with one isoprene unit (Q_1) in 20 mM potassium phosphate buffer, pH 8.0, containing 0.2% dodecyl maltoside, at 20 °C in a quartz cuvette with 1 cm light path equipped with a magnetic stirrer for effective temperature control in a cuvette holder temperature controlled by a circulating water system. The enzyme reaction was initiated by addition of 2−5 μL of the enzyme solution and followed by monitoring the absorbance decrease at 340 nm. The specific enzyme activity was determined by the initial slope after addition of the enzyme solution. Rotenone inhibition was determined at 1 μM inhibitor.

Quantitative Analyses of Non-Protein Constituents. (*1*) *FMN*. The isolated Complex I of 3 mg of protein was dissolved in 1 mL of 25 mL Tris-HCl buffer, pH 8.0, containing 0.1% dodecyl maltoside. The enzyme solution was treated with 5% trichloroacetic acid and homogenized with a Vortex mixer for 20 s. The protein portion was removed by centrifugation. The FMN content was determined by the absorbance difference between 445 and 580 nm using the molecular extinction coefficient of FMN, $\Delta\varepsilon_{445-580nm}$, of 10.9 mM^{-1} cm^{-1} in the presence of 5% trichloroacetic acid, which was determined by the present study using authentic FMN. The absorption spectrum of FMN is significantly dependent on pH. For example, $\Delta\varepsilon_{445-580nm}$ at pH 7.4 is 12.5 mM^{-1} cm^{-1}.

(*2*) *Q_{10}*. A 3 mg portion of the Complex I sample, placed in a small test tube, was mixed well with 1 mL of methanol with a Vortex mixer. Then, 1.5 mL of cyclohexane and 0.1 mL of H_2O were added and mixed vigorously with a Vortex mixer for 1 min. After centrifugation for 5 min at 3000 rpm, the cyclohexane layer was carefully taken up with a Pasteur pipet. Addition of 0.1 mL of H_2O in the above procedure is critical for the clear phase separation. The methanol layer was treated with two additional cyclohexane treatments for complete extraction of Q_{10}. The combined cyclohexane layers were dried under N_2 stream, followed by addition of 20 μL of cyclohexane and 500 μL of ethanol. After filtration of the Q_{10} solution with a 0.50 μm membrane filter, the 50 μL portion was applied to an HPLC system equipped with a C18 column (i.d. = 4.6 × 250 mm; Shiseido CAPC11 PAK column). Developing medium (ethanol/methanol/acetonitrile = 4/3/3) was introduced to the column equilibrated with the same medium at 1 mL/min at 45 °C. The Q_{10} isolated by the HPLC system was quantified by the peak area monitored at 275 nm. The specific peak area was determined by authentic Q_{10} solution, the concentration of which was evaluated by the molecular extinction coefficient of 14.02 $mM^{-1}cm^{-1}$ at 275 nm determined by the present work. No significant effect of UV light from the detector of the HPLC system was detectable on the spectrum of Q_{10}.

(*3*) *Inorganic Sulfur* (S^{2-}). The methylene blue method (*20*) was applied for the quantification as follows: 0.5 mL of zinc acetate solution (2.6% (w/v) $Zn^{2+}(CH_3COO^-)_2$ in water) was placed in a test tube which had been flushed with argon gas for sealing O_2. The enzyme solution (ca. 20 μL) at a concentration less than 60 μM containing 10% (v/v) glycerol was added carefully without significant turbulence in order for the enzyme solution giving a lower layer below the zinc acetate solution layer. Then, 0.1 mL of sodium hydroxide solution (6% (w/v) NaOH in water) was added, and the tube was sealed with a rubber septem, immediately followed by rigorous mixing for 1 min with a Vortex mixer. Then, the protein precipitate was removed by centrifugation (10000 rpm, 10 min). The supernatant was treated with 0.25 mL of 0.1% (w/v) *N*,*N*-dimethyl-*p*-phenylenediamine in 5 N HCl followed by the addition of 0.1 mL of 11.5 mM $FeCl_3$ in 0.6 N HCl. The test tube, sealed with a rubber septem, was shaken well with a Vortex mixer. The resulting solution was allowed to stand for 30 min at 20 °C. The methylene blue formed was quantified with the absorbance difference between 666 and 707 nm. The above procedure was repeated at five different amounts of a sample (0.25, 0.5, 0.75, 1.0, and 1.25 mg), and each $\Delta A_{666-707nm}$ was plotted against the amount of the sample treated. The content of S^{2-} in the sample was determined by comparing the standard curve determined by the authentic Na_2S solution. The standard solution of S^{2-} was prepared by dissolving $Na_2S \cdot 9H_2O$ in carefully deaerated H_2O in a test tube sealed with a septem and thoroughly flushed with N_2. The S^{2-} concentration in the standard solution was determined with the iodide titration.

(*4*) *P, Fe, and Zn*. Phosphorus was analyzed as follows: 0.5 mL of 60% perchloric acid solution was added to 0.2 mL of sample solution containing various amounts (2−6 mg) of the protein and kept overnight at 155 °C until a colorless transparent solution was obtained. After cooling the solution to room temperature, 2.4 mL of 0.22% ammonium molybdate and 0.1 mL of 1% amidol dissolved in 20% sodium bisulfate were

added, and the mixture was heated at 100 °C for 12 min. A glass ball was placed on the open end of the test tube for effective condensation of the water vapor during the above two heart treatments. Then, absorbance at 830 nm was determined (*21*). The phosphorus content was quantified by comparison of the slope of the plot of the absorbance at 830 nm against the amount of the protein with the slope for the standard sample (K_2HPO_4). The standard error for the present assay conditions for the complex was 2.5%. Iron and zinc contents were determined by ICP emission spectrometric analysis using Seiko Instruments Model SPS 4000.

RESULTS

Purification of Bovine Heart Complex I. The above purification procedure using 1000 g of minced bovine heart muscle provided approximately 12 g of protein of the mitochondrial membrane fraction. The deoxycholate treatment extracted 0.65 g of Complex I. The yields (percent) of the stepwise sucrose gradient centrifugation, the anion-exchange column chromatography, and the ammonium sulfate fractionation were 62, 22, and 18, respectively, compared with the amount of the enzyme activity extracted by the deoxycholate treatment. This purification procedure provided 0.12 ± 0.03 g (n = 24) of the final preparation in 2 days. No significant contaminant hemoproteins are detectable, consistent with the previous report (*22*). That is, the preparation is pure enough for determination of the enzyme concentration only from the absorption spectra. The SDS−PAGE pattern of the final preparation given in Figure 1A is consistent with the previously reported patterns (*12*).

Gel filtration chromatography using Sephacryl S-300 provided a single peak showing a molecular mass of 100 kDa as given in Figure 1B. No other minor peak is detectable. Electron microscopic analysis (the negative staining method) could be a powerful method for evaluation of the homogeneity and the integrity of purified proteins. The electron microscopic image of the present preparation of Complex I as given in Figure 1C shows that this preparation contains only L-shaped molecules with their sizes and shapes consistent with the previously reported images of Complex I molecules (*2*) and that no assembly or fragmentation of the L-shaped molecule is detectable. The preparation is homogeneous enough for single particle analysis which is under way.

Quantitative Determination of Non-Protein Constituents. (*1*) *FMN*. The standard error for five FMN determinations for 3 mg of a Complex I preparation by the present method was 1.6%, while the standard error for the protein determination by the Markwell method for six determinations was 7.1%. Thus, in this work, the enzyme concentration is given by the FMN content.

(*2*) *Q_{10}*. In the quantitative determination procedure as described above, the three time extraction of Q_{10} with cyclohexane was critical for quantitative extraction from the purified Complex I sample. The Q_{10} could be determined by its absorbance peak at 275 nm. However, dioctanoylphosphatidylcholine in 50 mM Tris-HCl buffer, pH 8.0, containing 0.16% decyl maltoside and 10% glycerol showed an absorption peak at 280 nm. In other words, the purified Complex I sample could contain phospholipids, the absorption spectrum of which overlaps that of Q_{10}. Thus, the HPLC treatment as described above is indispensable for accurate determination of Q_{10} content. The accuracy of the present Q_{10} determination was evaluated by 12 determinations for an identical Complex I preparation giving a standard error of 2.1%. Q_{10} content for 12 different batches of the enzyme provided a Q_{10}/FMN (mol/mol) ratio of 0.99 ± 0.23. The result is consistent with the ubiquinone content of *E. coli* Complex I, recently reported (*17*).

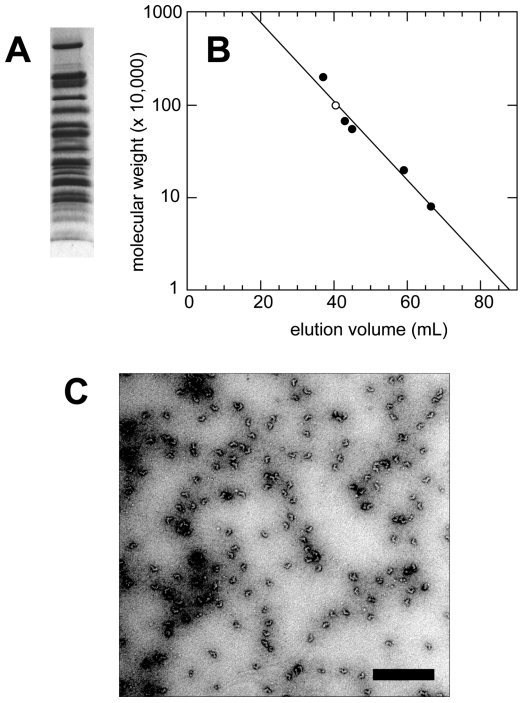

FIGURE 1: The size and shape of the purified bovine heart Complex I. (A) An SDS−PAGE pattern of the purified Complex I. Acrylamide concentration was 19%. (B) Gel filtration chromatography of the purified preparation using Sephacryl S-300 equilibrated with 40 mM Hepes-Na, pH 7.8, containing 100 M NaCl and 0.1% dodecyl maltoside. Filled circles denote the points of marker proteins as follows: blue dextran (2000000), thyroglobulin (669000), bovine heart F_oF_1-ATPase (550000), bovine heart cytochrome *c* oxidase (200000), and transferrin (80000). Open circle denotes the purified Complex I. (C) Electron microscopic image of the negatively stained Complex I purified with the present procedure. The electron microscope system used was JEM Model 1200. The scale bar = 200 nm.

(*3*) *Inorganic Sulfur* (S^{2-}). Careful avoidance of O_2 exposure to S^{2-} ion was most important for the quantitative determination. The standard error for the present method was 3.4%. The S^{2-}/FMN ratio was estimated to be 30.3 ± 2.4 from eight different batches of the preparation.

(*4*) *Metals*. Iron and zinc contents were not significantly influenced by EDTA treatment (dialysis for 6 h against 1 mM EDTA). Thus, these metals are unlikely to be adventitious contaminants. Iron and zinc per FMN were 30.6 ± 1.0 and 1.0 ± 0.1 for four determinations. No other metal was detectable.

(*5*) *Phosphorus*. The phosphorus per FMN was determined to be 71 ± 11 for 14 different batches of the preparation. The standard error of the present method was 2.5%. Thus, the standard deviation, 11 (15.5%), was induced by the inconsistency of the phosphorus content depending on the batch. The complete structures of all phospholipids (seven species) contained in Complex I have been determined by MS measurements including the chain length and the positions of the unsaturated bonds in the fatty acid (*23*). The composition of the phospholipids in Complex I was estimated by comparison of the intensity ratio of the mass spectra of Complex I determined by an ESI-TOF mass spectrometer (AccuTOF, JEOL, JMS-T100L) with those of bovine heart cytochrome *c* oxidase, the phospholipid ratio of which has been determined by the X-ray structure (*23*). The estimated

Table 1: Composition of Non-Protein Constituents of Bovine Heart NADH−Ubiquinone Reductase[a]

	mol/mol of FMN (SD)
Q_{10}	0.99 (0.23)
Zn	1.0 (0.1)
S	30.3 (2.4)
Fe	30.6 (1.0)
P	71 (11)

[a]Standard error for FMN determination: 1.6%.

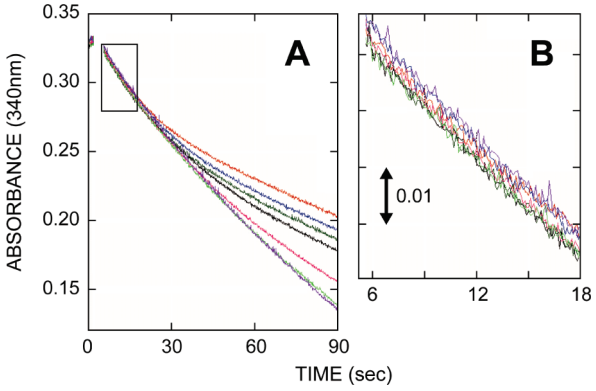

FIGURE 2: Effect of asolectin on NADH:Q_1 oxidoreductase activity of Complex I purified with the present procedure. Traces show the absorbance changes at 340 nm due to NADH oxidation after addition of 4.3 nM purified Complex I into the reaction mixture containing 150 μM NADH, 50 μM Q_1 in 20 mM potassium phosphate buffer, pH 8.0, 0.2% dodecyl maltoside, and various concentrations of asolectin (red, 0; blue, 4.9 μg/mL; green, 9.9 μg/mL; bluck, 20 μg/mL; pink, 39 μg/mL; light green, 78 μg/mL; purple, 120 μg/mL) at 30 °C. (A) Traces for about 90 s after the initiation of the enzyme reaction. (B) Initial traces in an expanded scale for the area defined by the square in panel A.

composition is 16 cardiolipins, 8 phosphatidylcholines, 23 phosphatidylethanolamines, and 8 phosphatidylglycerols.

The contents of non-protein constituents determined by the present work are summarized in Table 1. The standard deviations for determinations of Q_{10} and phosphorus atoms are significantly higher than the accuracy of each determination, while iron, sulfur, and FMN contents are significantly more consistent than those of Q_{10} and phosphorus atoms.

Enzyme Activity. The enzyme activity of the present preparation followed by NADH oxidation at 20 °C was 1.27 ± 0.31 μmol min^{-1} μmol^{-1} averaged for 14 different batches. The activity was increased at 30 °C up to 7 μmol min^{-1} μmol^{-1} (at 32 °C up to 8 μmol min^{-1} μmol^{-1}), which is comparable to the reported activity of the enzyme in mitochondria (*24*). In this paper, micromoles per minute per micromole is used for the unit of the enzyme activity. The unit corresponded to micromoles per minute per milligram of protein within the accuracy of the protein determination for the final enzyme preparation. It should be noted that these enzyme activities were detectable in the absence of any additional phospholipids. The enzyme activity decreased gradually during the course of NADH oxidation, giving a significant decrease in the slope with time as shown in Figure 2A. However, a strictly linear portion is detectable in the initial 12 s. The enzyme activity was determined from the linear portion. Addition of asolectin (up to 120 μg/mL) or other phospholipids did not influence the initial slope as shown in Figure 2B. However, as shown in Figure 2A, the extent of the spontaneous inactivation decreases with increasing concentration of asolectin. In the presence of 120 μg of asolectin/mL an essentially linear time course was obtained, suggesting removal of the spontaneous inactivation of Complex I. Rotenone inhibition of the enzyme activity was 90 ± 3%. Higher inhibition (99%) was detectable in the presence of decylubiquinone instead of Q_1.

The enzyme activity decrease during the NADH oxidation, which is eliminated by addition of phospholipids, might be induced by insufficient quinone dispersion which could limit the rate of access to the quinine binding site more strongly at lower Q_1 concentration. However, the insufficient quinone dispersion, if any, is unlikely to have any influence on the NADH oxidation by this enzyme, since the steady-state kinetic analyses of this enzyme show no such effect (*22*). That is, all of the relationships between NADH oxidation rate and Q concentration determined under various conditions are simple rectangular hyperbolic. Thus, in the Q concentration lower than K_m, NADH oxidation rate is proportional to the substrate concentration, indicating that quinine dispersion, if any, does not influence the NADH oxidation rate. Furthermore, the concentration range of Q_1 in the present reaction system is far lower than the solubility of Q_1.

DISCUSSION

Determination of the Non-Protein Intrinsic Constituents of Bovine Heart NADH−Ubiquinone Reductase. Since the discovery of Complex I almost 48 years ago (*16*), many purification methods have been reported giving various inconsistencies in the chemical constituents and functional properties (*9*−*15*). Purification, which is a prerequisite for elucidation of the reaction mechanism, has not been established for Complex I. It is desirable to include crystallization as the final step of purification of large multicomponent proteins. However, a reproducible purification method for a protein even without crystallization could provide essential information for composition of the intrinsic constituents of the protein and for the functional properties.

The present results strongly suggest that bovine heart complex I contains 1 FMN, 1Q_{10}, 30 Fe, 30 S^{2-}, 1 Zn^{2+}, and 70 phospholipids as given in Table 1. The iron/sulfur ratio of unity strongly suggests that all of the iron atoms are included in iron−sulfur clusters. The total number of iron atoms estimated by EPR analyses is 28, not 30. However, the experimental accuracies for the iron and sulfur determinations are not sufficiently high for concluding the presence of an extra iron−sulfur center besides the 8 centers thus far identified.

Although the physiological function is unknown, the reproducibility of the zinc content and the insensitivity to the EDTA treatment strongly suggest that zinc is one of the intrinsic constituents of Complex I, confirming the report by Giachini et al. (*25*).

The standard deviation of the phosphorus analyses (15.5% given in Table 1) is closely similar to that of the phosphorus analyses of the crystalline bovine heart cytochrome *c* oxidase (14.7%) (*23*). All of the phospholipids contained in the crystalline cytochrome *c* oxidase preparation detected by the phosphorus analysis are identified in the X-ray structure; that is, they are specifically bound to cytochrome *c* oxidase. The standard deviation for the phosphorus analyses of Complex I, closely similar to that for crystalline cytochrome *c* oxidase, suggests that all phospholipids found in Complex I by the phosphorus analysis are specifically bound to the protein moiety, as in the case of the phospholipids detectable in the crystalline cytochrome *c* oxidase

preparation. In other words, all of these 70 phospholipids are the intrinsic constituents of Complex I. No adventitious phospholipid is included in the present preparation. The conclusion is supported by the fact that further purification of the final preparation with the anion-exchange column with Poros-20HQ as described in Materials and Methods removed a significant amount of phospholipids concomitantly with decreasing the enzyme activity and the rotenone sensitivity, but without affecting the overall shape and size of the enzyme molecules in electron microscopic images and the blue native PAGE pattern.

Physiological Roles of Q_{10} and Phospholipids in Bovine Heart Complex I. As described above, the Complex I preparation obtained with the present procedure, which is fully active and rotenone sensitive, contains consistently one molecule of Q_{10}. The following facts strongly suggest that Q_{10} found in the purified preparation does not occupy the site for ubiquinone as the substrate: the stoichiometric binding of Q_{10} to the purified Complex I indicates that the affinity of Q_{10} is extremely high (K_d is essentially zero), since the purified Complex I preparation contains no significant Q_{10} free in the solution. On the other hand, the extensive steady-state kinetic analysis indicates that the Michaelis constant, K_m, for Q_1 is between 10 and 20 μM, depending on the concentrations of the other substrate (NADH) and the products (QH_2 and NAD^+) (*22*). One would expect that the long isoprene chain provides the extremely high affinity of Q_{10} to Complex I in the enzyme reaction system composed of the solubilized enzyme and the substrates (Q_1 and NADH) in aqueous solution where the highly hydrophobic Q_{10} is not released easily from the solubilized enzyme molecules to the aqueous phase. Then, the product $Q_{10}H_2$ with approximately the same overall polarity as that of Q_{10} is likely to have the affinity to the enzyme as high as that of Q_{10}. Thus, the Q_{10} and $Q_{10}H_2$ tightly bound to the substrate binding site would inactivate the enzyme seriously. The fact that the enzyme activity of the solubilized Complex I in the steady-state kinetic assay system is as high as the enzyme in the mitochondrial membrane strongly suggests that the tightly bound Q_{10} does not inhibit the enzyme activity.

As described above, Q_1 is accessible to the enzyme embedded in the mitochondrial membrane. Therefore, the long isoprene chain is not expected to contribute significantly to increase the substrate binding affinity. In other words, the affinity of Q_{10} to Complex I in the mitochondrial membrane is similar to that of Q_1 determined by the steady-state kinetic analysis. Therefore, these Q_{10} molecules at the substrate binding site, if any, would be readily removed during the isolation process.

Furthermore, a few preparations obtained by the present method contained fairly low Q_{10} (about 0.3 mol/enzyme), showing low enzyme activity (0.5 μmol min^{-1} μmol^{-1}). The phospholipid content and the overall size and shape of the preparations are consistent with those of the normal enzyme preparation containing a stoichiometric amount of Q_{10}. By replacing the dodecyl maltoside in the medium of the anion-exchange column chromatograph with Poros-20HQ in the present purification procedure with 6-cyclohexyl-1-hexyl-β-D-maltoside (Anatrace), the content of Q_{10} in the final preparation was decreased significantly to about 0.13 mol/enzyme concomitantly with enzyme activity decrease to 12% of that of the present enzyme preparation. These results also strongly suggest that Q_{10} is one of the cofactors of Complex I.

Rigorously speaking, the above results do not exclude the possibility that the bound Q_{10} with the long isoprene chain has only a structural role for stabilizing the conformation of the active site of the enzyme. However, in some enzyme preparations, Q_1 bound at the Q_{10} site provides normal enzyme function (*vide infra*). The results suggest that the bound Q_{10} is directly involved in the electron transfer.

As described in the previous section, the high consistency in the phospholipid content in our preparation suggests that all of these 70 phospholipids detectable in our preparation are specifically bound to Complex I. Removal of some phospholipids from our preparation results in lowering the enzyme activity and the rotenone sensitivity as described above. On the other hand, our Complex I preparation exerts full enzyme activity without addition of phospholipids in the reaction mixture for the enzyme activity assay. Addition of phospholipids does not influence the enzymatic activity as shown in the initial slope of the NADH oxidation (Figure 2B). These results strongly suggest that these 70 phospholipids are sufficient for keeping the conformation native. Preparations from other laboratories, which contain about 40 phospholipids, require phospholipids for full enzymatic activity (*9*−*15*), suggesting strongly that the externally added phospholipids are specifically bound to the phospholipid sites from which the intrinsic phospholipids have been removed during purification. Then, the conformation of the Q_{10} binding site is restored by the externally added phospholipids to have high affinity to Q_{10} or Q_{10} derivatives. In the enzyme assay system, Q_1 or decylubiquinone used as one of the substrates would be bound to the site for Q_{10} as the cofactor to exert the full activity.

It has been reported that addition of phospholipids in the ion-exchange chromatograph which is one of the purification steps in a Complex I preparation provided high enzyme activity in the presence of phospholipids in the reaction mixture of the enzyme activity assay, while significantly lower activity was obtained without addition of phospholipids in the chromatographic step (*15*). The Q_{10} content in the preparation is substoichiometric (0.3 ± 0.05 per Complex I) regardless of the enzyme activity. It has been proposed that, based on the above results, this enzyme has no intrinsic Q_{10}. However, the present results indicate that the following interpretation is preferable; the protective effect of phospholipids for spontaneous denaturation of Complex I, as shown in Figure 2A, avoids the denaturation of Complex I during the chromatography in the presence of phospholipids, so that the addition of phospholipids during enzyme assay restores the conformation of the Q-binding site to provide the full enzymatic activity. On the other hand, the chromatography in the absence of phospholipids induces an irreversible denaturation. Thus, the phospholipid addition cannot restore the enzyme activity.

As stated in the introduction, two Q_{10} sites giving SQ_{Nf} and SQ_{Ns} have been proposed (*5*, *18*). The binding mode must be greatly different for providing these different functions. The present Complex I preparation in the fully oxidized state contains one molecule of Q_{10}. If this Q_{10} binding site is one of the above proposed sites, the affinity of the other Q binding site must be much weaker and dependent on redox and/or membrane-potential dependent. Therefore, the Q_{10} found in the present preparation may function as the converter between one and two electron transfer pathways, since the converter should be tightly bound in any oxidation state. The affinity of the other site to Q_{10} could be controlled by the long-range interactions from the electron transfer pathway from the NADH binding site as has been suggested by the steady-state kinetic analyses (*22*).

The enzyme activity in the solubilized preparation was directly compared with that using the mitochondrial preparation under

turnover conditions. But this comparison should be considered just a first approximation, since the accessibility of Q_1 to the active site of the enzyme in the mitochondrial preparation must be significantly harder than that to the solubilized enzyme. The turnover activity of the solubilized Complex I is highly likely to be much higher than that of the mitochondrial preparation. Although the present preparation shows the enzyme activity as high as that of the mitochondrial preparation, further improvement of the purification method should be persuaded.

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