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The Ratio of Oxidative Phosphorylation Complexes I–V in Bovine Heart Mitochondria and the Composition of Respiratory Chain Supercomplexes*

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Hermann Schägger‡ and Kathy Pfeiffer

From the Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, D-60590 Frankfurt, Germany

The ratios of the oxidative phosphorylation complexes NADH:ubiquinone reductase (complex I), succinate:ubiquinone reductase (complex II), ubiquinol:cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV), and F_1F_0-ATP synthase (complex V) from bovine heart mitochondria were determined by applying three novel and independent approaches that gave consistent results: 1) a spectrophotometric-enzymatic assay making use of differential solubilization of complexes II and III and parallel assays of spectra and catalytic activities in the samples before and after ultracentrifugation were used for the determination of the ratios of complexes II, III, and IV; 2) an electrophoretic-densitometric approach using two-dimensional electrophoresis (blue native-polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis) and Coomassie blue-staining indices of subunits of complexes was used for determining the ratios of complexes I, III, IV, and V; and 3) two electrophoretic-densitometric approaches that are independent of the use of staining indices were used for determining the ratio of complexes I and III. For complexes I, II, III, IV, and V in bovine heart mitochondria, a ratio 1.1 ± 0.2:1.3 ± 0.1:3: 6.7 ± 0.8:3.5 ± 0.2 was determined.

The major components of the mammalian system of oxidative phosphorylation (OXPHOS)[1] are the four complexes of the respiratory chain, NADH:ubiquinone reductase (complex I), succinate:ubiquinone reductase (complex II), ubiquinol:cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV), and F_1F_0-ATP synthase (complex V). These complexes are functionally active when isolated as individual complexes. However, there is increasing evidence that complex V exists as a dimer in mitochondrial membranes and that the respiratory chain is a network of respiratory chain supercomplexes (1–3). The respiratory chain supercomplexes from bovine heart mitochondria, which were isolated with high yield using blue native-polyacrylamide gel electrophoresis (BN-PAGE), contained complex I in monomeric form, complex III in dimeric form, and a variable copy number of complex IV. Associations of complexes II and V with other complexes of the system of oxidative phosphorylation could not be identified under the conditions of BN-PAGE so far.

On the basis of a commonly used ratio for oxidative phosphorylation complexes I:II:III:IV:V of 1:2:3:6–7:3–5, which was published by Hatefi (4) in 1985, and a recent finding that complex I is almost quantitatively associated with complex III (2), a model for a network of mammalian respiratory chain complexes has been presented. According to this model, the respiratory chain exists as a mixture of two supercomplexes in a 2:1 ratio: a large supercomplex comprises complexes I, III, and IV; and a smaller supercomplex comprises complexes III and IV as well but is lacking complex I. However, the recovery of complex I after BN- and SDS-PAGE in a second dimension has not been tested in the previous work (2), and the ratio of OXPHOS complexes is not exactly known.

An often-cited review of the ratio of OXPHOS complexes by Capaldi *et al.* (5) gives concentration ranges of complexes per milligram of protein. Markedly differing complex I:II:III:IV:V ratios can be calculated from these data depending on the technique used for complex I determinations. A ratio of 1:1.5: 1.9–4.1:4.6–7.7:4–4.2 is calculated when complex I is quantified using FMN contents. These compositional data were rounded off to a 1:2:3:6:*x* ratio. However, a 1:3:3.8–8.2:9.2–15.2:8–8.4 ratio is calculated on the basis of original data sets using immunologic techniques to quantify complex I (5, 6). Different materials were used in the cited studies, such as bovine and rat mitochondria, Keilin-Hartree particles, and sonic particles, which may be a major reason for the large variations in the contents and ratios of the complexes.

The commonly used ratio of OXPHOS complexes in bovine sonic particles, which was published in a review article by Hatefi (4), was based on the content of FMN (complex I), covalently bound FAD (complex II), cytochrome c_1 (complex III), cytochrome aa_3 (complex IV), and F_1 (complex V) and adjusted to the nearest integer relative to complex I. Unfortunately, no S.D.s or ranges are given, except for complexes IV and V. The use of the FMN content in sonic particles as a single measure to quantify complex I seems critical. Complex I might have been overestimated if the sample contained FMN from another flavoprotein, and all ratios would need correction by a factor of 2 if complex I contained a second FMN, as postulated by Albracht and Hedderich (7).

Vinogradov and King (8) used Keilin-Hartree preparations to determine cytochromes, acid-extractable FMN and FAD, and acid-nonextractable flavin. Assuming that all acid-extractable FMN is bound to complex I (one FMN per complex I), and all acid-nonextractable flavin is part of complex II (one FAD per

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‡ To whom correspondence should be addressed: Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, Haus 25B, D-60590 Frankfurt, Germany. Tel.: 49-69-6301-6927; Fax: 49-69-6301-6970; E-mail: schaegger@zbc.klinik.uni-frankfurt.de.

[1] The abbreviations used are: OXPHOS, oxidative phosphorylation; BN, blue-native; PAGE, polyacrylamide gel electrophoresis; DDM, dodecylmaltoside; complex I, NADH:ubiquinone reductase; complex II, succinate:ubiquinone reductase; complex III, ubiquinol:cytochrome *c* reductase; complex IV, cytochrome *c* oxidase; complex V, F_1F_0-ATP synthase.

complex II), a ratio of complexes I:II:III:IV of 1:1.3:<2.7:5.6 can be calculated from their data. The value for complex III is uncertain, because no correction for interfering cytochrome *b* of complex II was made. Assuming that, in contrast to sonic particles, 20% of total FMN of Keilin-Hartree particles is not part of complex I gives a complex I:II:III:IV ratio of 1:1.6:<3.4:7, which is close to the commonly used ratio. Again the critical FMN content is the only measure to quantify complex I, and novel approaches not relying on the assumptions concerning FMN and FAD contents seem to be required to obtain reliable ratios of OXPHOS complexes.

Knowing the accurate ratios of respiratory chain complexes is essential for the correct interpretation of kinetic and spectroscopic data obtained with native mitochondrial membranes, because the type and number of supercomplexes and the fraction of free individual complexes will define the overall throughput rates of the respiratory chain. Knowing the accurate ratios and the structural organization of the building blocks of the respiratory chain is also essential to evaluate the importance of substrate channeling *versus* pool function of quinone and cytochrome *c* (see "Discussion"). Therefore, we reassessed the stoichiometry of the respiratory chain complexes, in particular the complex I:III ratio, by using three novel and independent approaches.

EXPERIMENTAL PROCEDURES

Materials—Imidazole was obtained from Fluka; dodecylmaltoside was from Biomol; and decylquinazolinamine was a kind gift from Aventis Crop Science (Frankfurt, Germany). Inhibitors and all other chemicals were purchased from Sigma.

Isolation of Mitochondria and Oxidative Phosphorylation Complexes—Bovine heart mitochondria were prepared according to the method of Smith (9). Complex I, which contains one FMN per monomer, was prepared as described by Okun *et al.* (10). The FMN content was 0.99 nmol of FMN/mg of protein. Complex III, which contains two heme b per monomer or one cytochrome c_1 per monomer, was isolated according to the method of Schägger *et al.* (11). The cytochrome *b* content of the preparation used was 3.5 nmol/mg. Complex IV was prepared according to the method of Brandt *et al.* (12). The preparation used had a cytochrome *a* content of 9.8 nmol/mg of protein. Complex V was prepared as described elsewhere.[2]

Spectrophotometric Measurements and Absorption Coefficients—Difference spectra of dithionite-reduced minus ferricyanide-oxidized samples were recorded in the range of 650–500 nm using a Shimadzu UV 300 spectrophotometer. The following absorption coefficients were used: cytochrome *b* of complex II, $\epsilon_{561-575\ nm} = 26\ mM^{-1}cm^{-1}$; cytochromes b_L and b_H of complex III, $\epsilon_{562-575\ nm} = 2 \times 28.5\ mM^{-1}cm^{-1}$; and cytochrome $a + a_3$ of complex IV, $\epsilon_{603-630\ nm} = 24\ mM^{-1}cm^{-1}$.

Catalytic Activities—NADH:ubiquinol reductase activity of complex I was measured at pH 8, 30 °C, as decylquinazolinamine-sensitive oxidation of NADH (200 μM, 340–400 nm; $\epsilon = 3.4\ mM^{-1}cm^{-1}$) using 75 μM decylbenzoquinone as electron acceptor. Succinate:dichloroindophenol reductase activity of complex II was measured at pH 7, 30 °C, as thenoyltrifluoroacetone-sensitive reduction of dichloroindophenol (0.002%, 610–750 nm; $\epsilon = 20.5\ mM^{-1}cm^{-1}$) using 50 mM succinate as a substrate. Ubiquinol:cytochrome *c* reductase activity of complex III and cytochrome *c* oxidase activity of complex IV were measured at 25 °C, pH 7.2, by the antimycin-sensitive reduction and cyanide-sensitive oxidation of cytochrome *c*, respectively (70 μM cytochrome *c*, 550–540 nm; $\epsilon = 19.1\ mM^{-1}cm^{-1}$). Decylbenzoquinol (75 μM) was used as substrate for cytochrome *c* reductase. Oligomycin-sensitive ATP hydrolysis of complex V was measured at 25 °C using an assay coupled to the oxidation of NADH. Shortly before the test, 0.25 mM NADH, 1 mM phosphoenolpyruvate, 2.5 units/ml lactate dehydrogenase, and 2 units/ml pyruvate kinase were added to the test buffer (250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 2 mM NaCN, and 20 mM Tris/HCl, pH 7.5).

Electrophoretic Techniques—Tricine-SDS-PAGE, BN-PAGE, and two-dimensional PAGE (BN-PAGE/SDS-PAGE; Ref. 13), three-dimensional PAGE (BN-PAGE/modified BN-PAGE/SDS-PAGE; ref. 2), Coomassie blue staining, and densitometric protein quantification (14) were performed as described.

[2] H. Schägger, unpublished procedures.

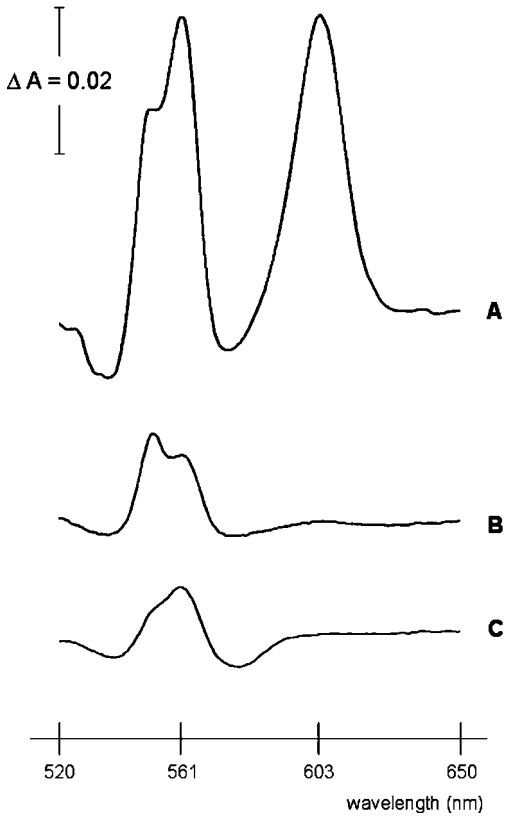

FIG. 1. **Difference spectra (reduced minus oxidized) of samples used for determining the ratio of complexes II:III:IV.** *A*, dithionite-reduced minus ferricyanide-oxidized spectrum of solubilized bovine heart mitochondria. Cytochromes *b* of complexes II and III contribute to the absorption at 561 nm. *B*, complex II-containing supernatant after selective solubilization of mitochondria and ultracentrifugation. The absorption at 561 nm is mostly attributable to complex II, because the supernatant contains almost all complex II activity and very low complex III activity (see "Results"). *C*, complex II supernatant was used as in *B*, but cyanide was added to the reference cuvette instead of ferricyanide. Endosubstrates reduced cytochrome *c* and complex IV in the reference cuvette, and a typical spectrum of complex II was obtained, which is characterized by cytochrome *b* absorption with a short wavelength shoulder.

RESULTS

Determination of the Ratio of Complexes II:III:IV (Method 1)—The spectrophotometric-enzymatic assay described here for determining the molar ratio of complexes II, III, and IV made use of a differential solubilization of complexes II and III and parallel assays of spectra and catalytic activities in the samples before and after ultracentrifugation.

Bovine heart mitochondria were suspended in 100 mM NaCl and 20 mM imidazole, pH 7, and partially solubilized by adding Triton X-100 (Triton X-100:protein ratios from 1.0 to 1.4 g/g; final protein concentration, 8.8 mg/ml). Half of each sample was saved as a reference. The second half was centrifuged for 30 min at 100,000 × *g*. Succinate:dichloroindophenol reductase activity of complex II was recovered quantitatively in all supernatants, but decylbenzoquinol:cytochrome *c* reductase activity of complex III was only 2.3% using a Triton X-100:protein ratio of 1 g/g and 10% using 1.4 g/g. Fractions of 19 and 24.3%, respectively, of the total cytochrome *b* absorption of bovine heart mitochondria (dithionite-ferricyanide, 562–575 nm) were found in the supernatants (Fig. 1, *A* and *B*). Knowing the percentages of complexes II and III recovered in the supernatants and the extinction coefficients for the *b*-type cytochromes of isolated complexes II and III, the contribution of complex II to the total heme b absorption in bovine heart mitochondria was calculated as 17.1 and 15.9% from these two experiments

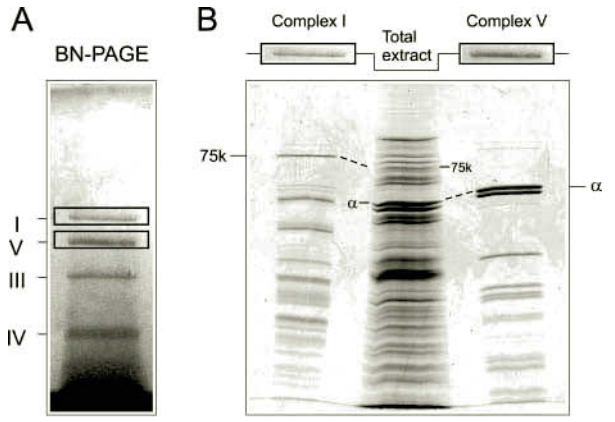

FIG. 2. **Recovery of complexes I and V after BN-PAGE.** *A*, OXPHOS complexes were solubilized from 70 μg of bovine heart mitochondrial protein using DDM and separated by BN-PAGE. *B*, The bands of complexes I and V were then excised and loaded onto an 8% acrylamide Tricine-SDS gel. The same amount of mitochondrial protein was solubilized directly by SDS and loaded onto the same gel. The migration distances of identical proteins from the isolated complexes and from the total extract differ, because the two types of samples differed considerably in ionic strength. The 75-kDa band of complex I was identified by Western blotting (data not shown).

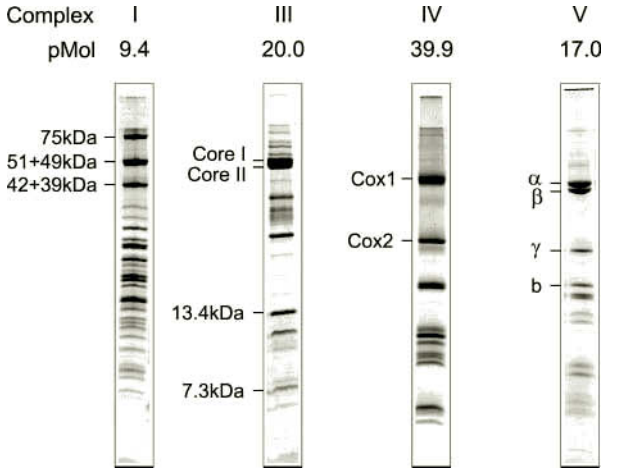

FIG. 3. **Purity and subunit composition of OXPHOS complexes used for determination of Coomassie blue staining indices of subunits.** Defined amounts of FMN in isolated complex I, cytochrome *b* of complex III, cytochrome *a* of complex IV, and complex V protein were loaded to Tricine-SDS gels (16.5% acrylamide gels for complexes I, III, and IV and a 10% acrylamide gel for complex V). Coomassie blue staining indices were determined for the assigned bands and subunits (see Table I).

(16.6 ± 0.6% from five different experiments), and the ratio of complex II:III was determined as 1:2.3 ± 0.1.

Complex III and IV concentrations were calculated from dithionite minus ferricyanide redox spectra (Fig. 1*A*). After subtracting 16.6% of the total heme b absorption attributable to complex II, a complex III:IV ratio of 1:2.2 ± 0.2 (n = 8) was calculated. The overall ratio of complexes II:III:IV determined by this approach was 1.3 ± 0.1:3:6.6 ± 0.6 (normalized to complex III = 3). These ratios refer to monomeric complexes independent of their physiological oligomerization states.

Ratio of Complexes I, III, IV, and V (Method 2)—A first electrophoretic-densitometric approach to measure the stoichiometry of OXPHOS complexes made use of a near quantitative solubilization of all OXPHOS complexes from bovine heart mitochondria by dodecylmaltoside, followed by separation of the complexes by BN-PAGE, separation of the subunits by SDS-PAGE in a second dimension, and densitometric quantification of selected subunits after Coomassie blue staining. Near quantitative solubilization and recovery of the complexes after BN-PAGE was a prerequisite for this approach, and the staining indices of individual subunits of complexes had to be determined.

Recovery of OXPHOS Complexes after Solubilization by Dodecylmaltoside—Bovine heart mitochondria were solubilized under conditions usually used for BN-PAGE but on a larger scale. Sedimented mitochondria (20 mg of protein) were suspended in 2 ml of 50 mM NaCl and 50 mM imidazole/HCl, pH 7, and solubilized by addition of dodecylmaltoside (DDM:protein ratio, 1.6 g/g). Half was kept as a reference. The second half was centrifuged 30 min at 100,000 × g. Dithionite minus ferricyanide difference spectra and catalytic activities were measured using the sample before centrifugation and the supernatant after centrifugation. The recovery of all complexes was between 95 and 100%.

Recovery of OXPHOS Complexes after BN-PAGE—Bovine heart mitochondria were solubilized for BN-PAGE as described above using a DDM:protein ratio of 1.6 g/g. An aliquot of the supernatant was used for BN-PAGE (70 μg of total protein applied to a 2-cm sample well). After BN-PAGE, the two bands of complexes I and V were excised, as indicated in Fig. 2*A* and placed on top of an 8% acrylamide Tricine-SDS gel (Fig. 2*B*). Another 70-μg aliquot of the supernatant after ultracentrifugation was supplemented with SDS and directly loaded onto the same 8% acrylamide gel as a 100% reference. After electrophoretic separation and Coomassie blue staining, the staining intensities of the 75-kDa band of isolated complex I and of the α subunit of isolated complex V were densitometrically compared with the corresponding bands of the reference lane (Fig. 2*B*). The recoveries from the BN gel were 92 ± 8% for complex I and 81 ± 11% for complex V (n = 5). The 75-kDa band was identified immunologically as the upper band of a doublet (data not shown). Potential contaminations of this band by proteins of similar size seem to be negligible, because this would lower the apparent recovery from BN gels, and the measured near quantitative recovery would not be attained. The α subunit, but not the β subunit, was used to quantify the recovery of complex V, because the apparent recovery using the α subunit was ~10% higher, indicating less contamination. Recoveries of complexes III and IV have been analyzed by using a different approach. Before the sample was applied to BN-PAGE, complexes III and IV were quantified spectrophotometrically. After BN-PAGE, the two bands were excised and resolved by SDS-PAGE using defined amounts of chromatographically purified complexes III and IV as standards in the same gel. The yield of complex III was ~95% with complex III and >80% with complex IV, which gives rise to streaking bands during BN-PAGE (14).

Determination of Staining Indices—Defined amounts of isolated complexes I–V, usually in the 5–60-pmol range, were applied to SDS gels, and the subunits were quantified after Coomassie blue staining using a laser densitometer. One example of an SDS gel used for determination of staining indices is shown in Fig. 3. The staining intensities (arbitrary units) of the subunits listed in Table I were normalized to 1 pmol and 1 kDa and finally normalized to the core I and II subunits of complex III, which were used as standards in each gel.

One of the two-dimensional gels used for determination of the ratios of complexes in bovine heart mitochondria is shown in Fig. 4*A*. A complex I:III:IV:V ratio (normalized to complex III = 3) of 1.2 ± 0.1:3:5.9 ± 0.6:3.1 ± 0.1 (n = 4) was determined this way. Taking into account the low recovery of complexes IV and V after BN-PAGE, which is ~10–15% lower than with complexes I and III, the complex I:III:IV:V ratio is 1.1–1.3:3:5.9–7.5:3.3–3.7. This electrophoretic approach using

TABLE I
Normalized Coomassie blue-staining indices of subunits of OXPHOS complexes

Coomassie blue-staining intensities (arbitrary units) of individual subunits were normalized to 1 kDa and 1 pmol of the complex. Finally the staining indices were normalized to the staining of core proteins I and II of complex III, which were used as standards in each gel.

Complex	Subunit	Mass	Staining index (n = 7)
		kDa	
I	75 kDa	77	1.02 ± 0.15
	51 + 49 kDa	98	1.19 ± 0.28
	42 + 39 kDa	76	1.37 ± 0.21
III	Core protein 1	49	0.92 ± 0.08
	Core protein 2	47	1.08 ± 0.09
	13.4 kDa	13.4	1.70 ± 0.15
	7.3 kDa	7.3	1.44 ± 0.13
IV	Cox subunit 1	57	0.54 ± 0.05
	Cox subunit 2	26	0.76 ± 0.07
V	α subunit	55	0.94 ± 0.07
	β subunit	52	0.91 ± 0.07
	γ subunit	30	1.43 ± 0.12
	Subunit b	25	1.24 ± 0.10

staining indices for individual subunits (Table I) was also applied to bovine heart submitochondrial particles. The ratio of complexes I:III:IV:V was found to be almost identical to the ratio determined for mitochondria (data not shown).

Determination of the Complex I:III Ratio (Method 3A)—A second electrophoretic-densitometric approach started with the solubilization procedure described above, but digitonin was used instead of dodecylmaltoside (digitonin:protein ratio, 4–8 g/g) to retain respiratory chain supercomplexes. The recoveries of complexes in the supernatant after centrifugation using ezymatic assays were 86 ± 1% for complex I, 100% for complex II, 87 ± 4% for complex III, 91 ± 5% for complex IV, and 86 ± 3% for complex V, which compares well with the solubilization by dodecylmaltoside.

BN-PAGE was then used to separate the supercomplexes and individual OXPHOS complexes, and a second-dimension SDS-PAGE followed to resolve the subunits (Fig. 4*B*). Some of the Coomassie blue-stained subunits were selected for densitometric quantification, *e.g.* the 75-, 51 + 49-, and 42 + 39-kDa bands of complex I and the core proteins 1 and 2 of complex III. The identity of these bands was verified by amino-terminal protein sequencing. The total staining intensities of each of these specific subunits in supercomplexes and free complexes was summed (Fig. 4*B*) and compared with the signal intensities in a reference two-dimensional gel from a dodecylmaltoside-solubilized sample (Fig. 4*A*). The recoveries of digitonin-solubilized complexes compared with those of dodecylmaltoside-solubilized complexes were 103 ± 24% for complex I, 100 ± 12% for complex II, 104 ± 23% for complex III, >87% for free complex IV (complex IV bound to supercomplexes could not be reliably quantified), and 95 ± 13% for complex V (n = 5). This indicated also that the recovery of digitonin-solubilized complexes was near quantitative, and it was possible to determine the ratio of complexes I and III in a way that was independent of the staining indices of individual subunits.

Using two-dimensional gels of digitonin-solubilized mitochondrial complexes, we found that 86 ± 1.5% of total complex I and 60 ± 9.5% of total complex III (n = 4) were bound to supercomplexes, whereas 14 ± 1.5% and 40 ± 9.5%, respectively, existed as free individual complexes I and III. All supercomplexes contain a complex I monomer, which binds to a complex III dimer, and a variable complex IV copy number, as illustrated in Fig. 5. This means that 86% of total complex I and 60% of total complex III are fixed in a 1:2 ratio; 100% complex I monomer would then fix 70% of total complex III and leave 30% in free form. Therefore, the complex I:III ratio determined this way is ~0.8–1.2:3.

Determination of the Complex I:III Ratio (Method 3B)—A third electrophoretic-densitometric approach to determine the complex I:III ratio started with the solubilization procedures using digitonin and dodecylmaltoside as described above, but after first-dimension BN-PAGE, a second modified BN-PAGE followed using low detergent in the cathode buffer. The rationale behind this approach was to improve reliability of the quantification, because the core protein I subunit of complex III and the 51 + 49-kDa band of complex I were rather close in the two-dimensional gel (Fig. 4*B*).

In two-dimensional native gels, which are native in both dimensions (BN-PAGE/modified BN-PAGE), all complexes that exist as individual complexes in first BN-PAGE are found on a diagonal in the second-dimension gel. This is shown in Fig. 6*A,* in which dodecylmaltoside was used for solubilization and separation of isolated complexes. However, solubilization by digitonin led to separated supercomplexes in the first-dimension BN-PAGE, which dissociated into the individual complexes in the second-dimension BN-PAGE and therefore were found below this diagonal (Fig. 6*B*). Horizontal lines of individual complexes, *e.g.* complexes I and III, emerged, which were excised and put on top of third-dimension SDS gels (Fig. 6, *C* and *E*). Gel pieces containing complexes I and III from DDM-solubilized samples (Fig. 6*A*) were applied to the same gels and were used as references for quantitative recovery. Using this approach, it was possible to use any subunit of a complex for densitometric quantification except those that were detergent-labile and dissociated easily, such as the iron-sulfur protein of complex III. Also excluded were those subunits that were not clearly separated from contaminating proteins, *e.g.* the 51 + 49-kDa band of complex I, which was too close to the β subunit of complex V (Fig. 6*C*).

Using digitonin-solubilized mitochondria and the three-dimensional approach, we measured that 84 ± 1.2% of total complex I and 58 ± 3.2% of total complex III (n = 4) were fixed in supercomplexes, whereas 16 ± 1.2% and 42 ± 3.2%, respectively, were present in free form; 100% complex I monomer would then fix 69% of total complex III and leave 31% in free form. The complex I:III ratio determined this way was 1.0–1.1:3. All ratios determined in this work are summarized in Table II.

DISCUSSION

The focus of this work was to determine the accurate complex I:III ratio in bovine heart mitochondria by novel and independent approaches to obtain a safe basis for a recent structural model of the mammalian respirasome (2) and its mechanistic implications.

A 1:3 ratio of complex I:III, which is mostly used, has been determined by using optical spectroscopy after extraction of FMN and heme from sonic particles (4). However, recent data suggested that complex I might have been overestimated because of contaminating FMN, because a 1:10 ratio of complex I:IV corresponding to a 1:4.3–5 ratio of complexes I:III was determined by fluorescent quench techniques (10). Both assays were based on the assumption that membrane-integrated complex I binds one FMN molecule, as does isolated complex I (10). In contrast to this assumption, a second FMN, which can be lost from complex I during isolation, was postulated to explain EPR spectra of complex I (7). This could explain why Smith *et al.* (6) determined an FMN content of 0.157 nmol/mg of bovine sonic particles, whereas immunological assays indicated a complex I content of 0.066–0.075. If the assumption of two molecules of FMN per complex I were correct, almost all previously

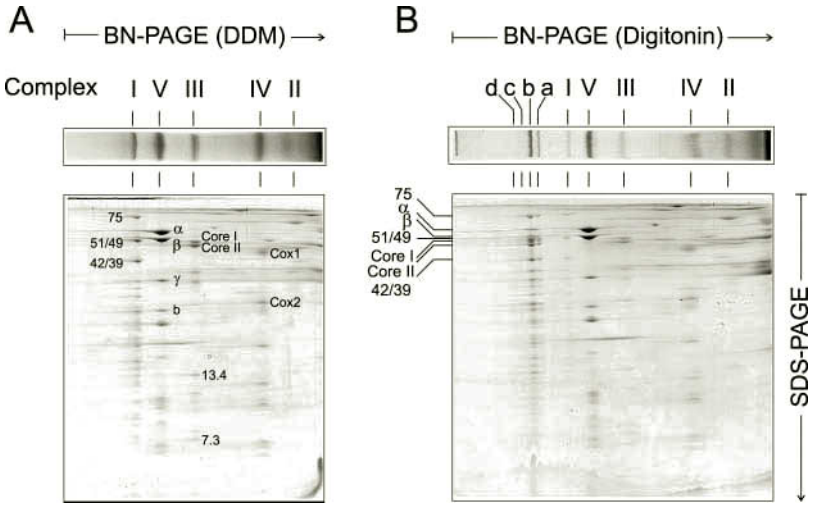

FIG. 4. **Two-dimensional resolution of OXPHOS complexes and supercomplexes for determining the complex I:III ratio.** The OXPHOS complexes and supercomplexes (*a–d*) from bovine heart mitochondria were solubilized using dodecylmaltoside (*A*) and digitonin (*B*) and separated by BN-PAGE. The ratio of complexes I:III:IV:V was determined from a two-dimensional gel (*A*) using the Coomassie blue-staining indices of the assigned subunits (Table I). A two-dimensional-gel (*B*) was used for determining the fractions of complexes I and III in free form and bound to supercomplexes, respectively, and for determining the complex I:III ratio according to method 3A (see "Results").

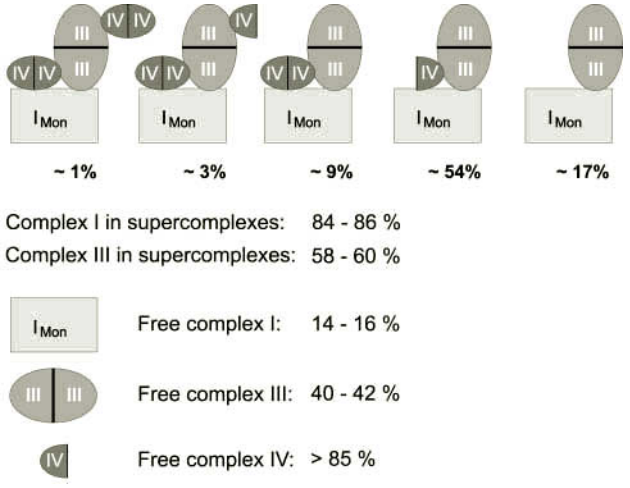

FIG. 5. **Distribution of respiratory chain complexes after solubilization of bovine heart mitochondria by digitonin.** Almost all monomeric complex I of bovine heart mitochondria (I_{Mon}) is bound to complex III to form various supercomplexes, which all contain a complex I monomer, a complex III dimer, and a variable copy number of complex IV. The major supercomplex, which represents >50% of total complex I of mitochondria, contains one copy of complex IV, which is stabilized by binding to complexes I and III (2). The percentages of individual respirasome fragments were determined by using methods 3A and 3B.

published data on mitochondrial complex I contents would need correction by a factor of 2.

We used three novel electrophoretic-densitometric approaches to determine the complex I:III ratio. Isolated complex I containing one FMN was used for determining the Coomassie blue-staining ratios of subunits according to method 2, whereas methods 3A and 3B were independent of the FMN content of complex I. The result, an average complex I:III ratio of 1.1 ± 0.2:3, is close to the 1:3 ratio published by Hatefi (4), but it is more than a simple confirmation by using independent techniques. Because the result obtained in this work is close to the ratio after FMN extraction from sonic particles, we conclude that there is no evidence for a second FMN of complex I, as suggested by Albracht and Hedderich (7) and Smith *et al.* (6). Also, contaminating FMN seems not to be a problem with the extraction of sonic particles. We conclude that membrane-integrated complex I contains one FMN, like isolated complex I, and that FMN is not bound in significant amounts to a mitochondrial membrane protein other than complex I.

Knowing the accurate stoichiometry of oxidative phosphorylation complexes is necessary to develop plausible structural models of the mammalian "respirasome" with testable mechanistic implications. Complex I-III interactions are rather stable, and only 14–16% of total complex I was found in free form in the presence of digitonin. So it seems likely that all complex I is bound to complex III in the absence of digitonin, but approximately one-third of total complex III is not bound to complex I at the measured 1.1 ± 0.2:3 ratio of complexes I:III. This ratio is compatible with a model of the respirasome consisting of large and small supercomplexes in a 2:1 ratio (Ref. 2 and Fig. 7). Stable binding of complex I to complex III opens the possibility for quinone substrate channeling, which would make a reaction independent of the bulk properties of a substrate pool. A quinone pool function has been shown in mammalian mitochondria (15), but the question arises of how important the quinone pool function for the electron transfer between complexes I and III would be. Assuming that binding of complex I to complex III does not alter the access to the quinone pool, complex II and other FAD-dependent dehydrogenases such glycerolphosphate dehydrogenase and electron-transferring flavoprotein dehydrogenase would have access to all complex III, including also the fraction of complex III that is integrated into the large supercomplexes. However, if binding of complex I to complex III segregates them from the quinone pool, complex II and other FAD-dependent dehydrogenases would only have access to the smaller supercomplex. NADH and $FADH_2$ respiration should then involve distinct subsets of complexes III and IV. Using this as a working hypothesis, we have now started new studies on the importance of quinone pool function *versus* substrate channeling for specific NADH and $FADH_2$ oxidation pathways.

A second major objective of this work was to obtain accurate stoichiometric data for complex II. Hatefi (4) measured acid-extractable FMN and acid-nonextractable FAD to determine the complex I:II ratio and found a complex I:II ratio of 1:2 for bovine sonic particles. We used an FMN- and a FAD-independent spectrophotometric-enzymatic approach. The ratio of complexes II:III usually is difficult to determine by spectrophotometric approaches, because the two complexes contain *b*-type cytochromes with similar spectra. However, we made use of the special solubilization properties of Triton X-100 with bovine mitochondrial membranes. Triton X-100 can solubilize complexes II and V selectively using low Triton X-100:protein ratios, because complex I-III-IV interactions are retained under these conditions, and the supercomplexes seem to be further linked together to form an extensive network of supercomplexes (2). Selective solubilization of complex II, combined with spectral and enzymatic quantifications, alleviated experimental problems considerably and minimized the experimental

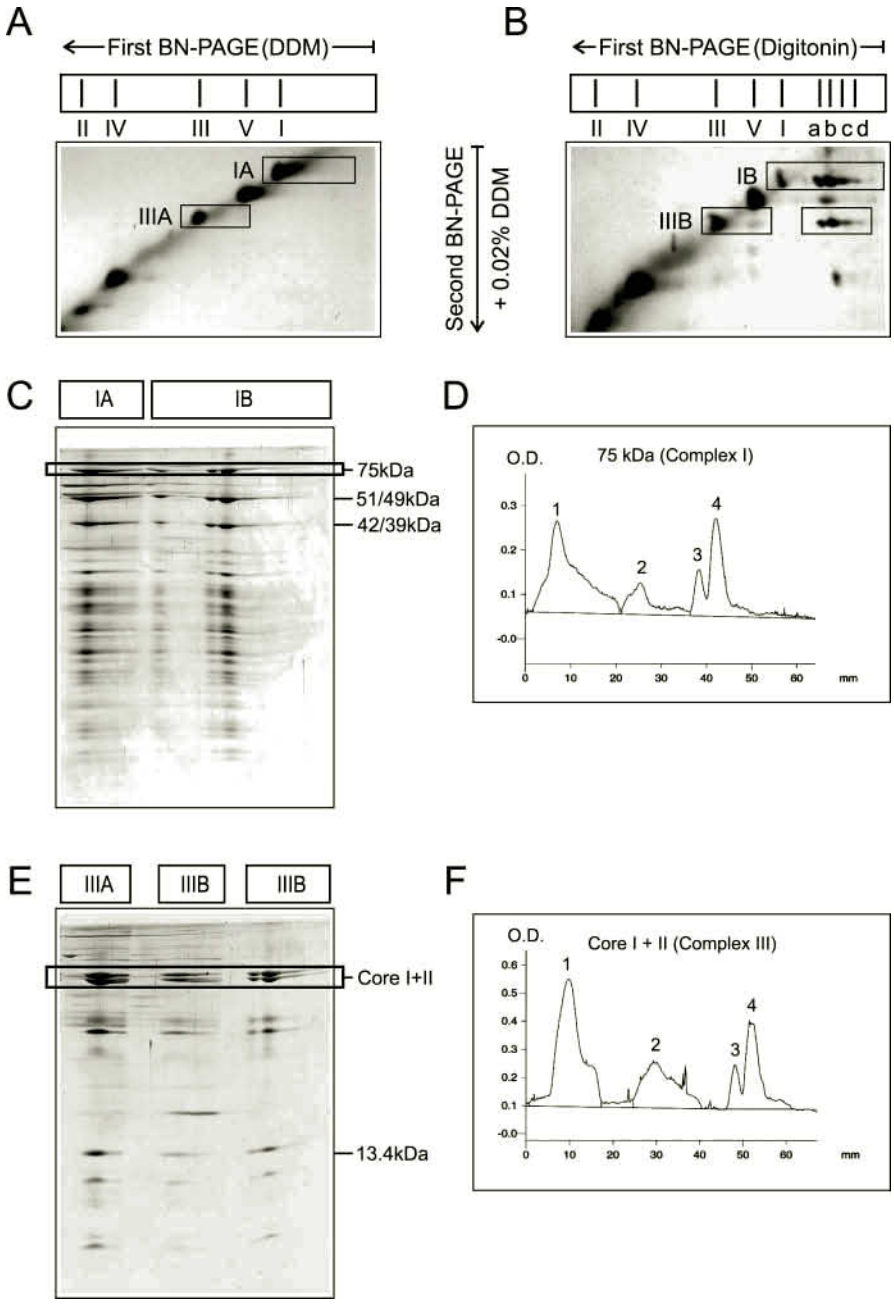

FIG. 6. **Three-dimensional resolution of OXPHOS complexes and supercomplexes for determining the complex I:III ratio.** OXPHOS complexes and supercomplexes from bovine heart mitochondria were solubilized and separated by a first BN-PAGE as in Fig. 4, *A* and *B*, followed by a modified BN-PAGE in a second dimension. *A*, dodecylmaltoside-solubilized complexes exist as individual complexes after the first and second BN-PAGE and are therefore found on a *diagonal* in the two-dimensional-gel. *B*, Digitonin-solubilized supercomplexes from the first BN-PAGE, however, are dissociated into individual complexes by the second modified BN-PAGE and are found below the *diagonal*. *C* and *E*, Gel pieces from *A* and *B* containing complexes I and III were loaded onto gels for a third-dimension SDS-PAGE. Selected subunits, as indicated, were used for the densitometric quantification of complexes: *D, peak 1*, and *F, peak 1*, from a DDM-solubilized sample, represent staining intensities of complexes I and III, respectively, which were used as references for quantitative recovery. *D, peaks 2–4*, and *F, peaks 2–4*, represent fractions of complex I and complex III, respectively, from a digitonin-solubilized sample. *D, 2*, and *F, 2,* fractions of free individual complexes I and III, respectively. *D, 3*, and *F, 3,* fractions of complexes I and III, respectively, bound to a supercomplex containing a complex I monomer and a complex III dimer. *D, 4*, and *F, 4,* fractions of complexes I and III, respectively, bound to a similar supercomplex containing one copy of complex IV in addition.

TABLE II
Comparison of published ratios of OXPHOS complexes in bovine heart mitochondria

The ratios were normalized to complex III = 3.

	Complex				
	I	II	III	IV	V
Hatefi (4)	1	2	3	6–7	3–5
This work					
Method 1		1.2–1.4	3	6.0–7.2	
Method 2	1.1–1.3		3	5.9–7.5	3.3–3.7
Method 3A	0.8–1.2		3		
Method 3B	1.0–1.1		3		

error for the determination of the complex II:III ratio (1.3 ± 0.1:3). The result is not compatible with the commonly used 2:3 ratio given by Hatefi (4). Complex II is clearly substoichiometric to complex III, but the ratio of monomeric complex II to dimeric complex III is close to 1:1. We cannot exclude that there might exist a specific association of the two complexes, although such an assembly could not be detected by BN-PAGE. The ratio of FAD and cytochrome c_1 in isolated succinate cytochrome *c* reductase is different: It is 1:1; *i.e.* two copies of complex II are bound to one complex III dimer (16).

We determined a complex III:IV ratio of 3:5.9–7.5 using a spectrophotometric approach and an independent electrophoretic-densitometric approach. This result confirmed previous analyses (4, 17).

Bertina *et al.* (18) quantified, the binding of inhibitors to complexes III and V in rat heart and liver mitochondria. The

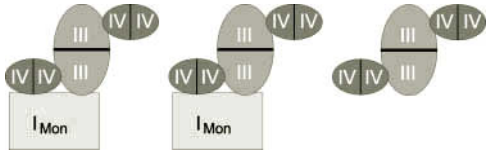

FIG. 7. **Model for the structure of the respirasome of bovine heart mitochondria.** This model was initially depicted on the basis of the identification of direct interactions of complexes (2) and on the overall 1:3:6 stoichiometry of complexes I:III:IV determined by Hatefi (4). It postulates that two major building blocks, which exist in a 2:1 ratio, are the constituents of the respiratory chain, namely, a large supercomplex containing a complex I monomer, a complex III dimer, and two complex IV dimers and a small supercomplex also containing complexes III and IV but missing complex I. The structural basis for this model, *i.e.* a ratio of 1.1 ± 0.2:3:6.7 ± 0.8 for complexes I:III:IV, and an almost quantitative binding of complex I to complex III despite the presence of digitonin, was verified in this work. Quantitative binding of complex IV to complex III and stable interactions of complexes II and III are still speculative and seem to require analyses in the absence of detergents. I_{Mon}, monomeric complex I of bovine heart mitochondria.

binding of anti-mycin to heart complex III (0.26 μmol/g) was almost identical to the binding of oligomycin and aurovertin (0.27 μmol/g each). This indicated a 1:1 ratio of complexes III and V, which essentially matches the 3:3.3–3.7 ratio that we found using an independent electrophoretic-densitometric approach. Almost identical results have also been obtained using immunological techniques for determining the F_1 content in sonic particles (19, 20). F_1 contents were in the range from 0.38–0.45 nmol/mg, which is equivalent to a complex III:IV ratio of 1:1–1.1.

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