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Subunit Analysis of Bovine Cytochrome bc_1 by Reverse-Phase HPLC and Determination of the Subunit Molecular Masses by Electrospray Ionization Mass Spectrometry†

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ABSTRACT: A sensitive and simple scheme was developed for the rapid separation of mitochondrial complex III subunits by reverse-phase high-performance liquid chromatography (reverse-phase HPLC). Ten of the 11 subunits of cytochrome bc_1 complex were separated with nearly baseline resolution between each peak. Cytochrome *b* was precipitated by acetonitrile on the column and could not be analyzed; the 10 other polypeptides were positively identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrospray ionization mass spectrometry (ESI/MS). The ESI/MS-determined molecular masses for subunits II, VI, VIII, IX, and XI are in excellent agreement with previously reported values; i.e., all are within ±2 mass units per 10 kDa. None of the other subunits gave molecular masses that agree with the published sequence values. The molecular mass of subunit I is 49 236 Da, which is far greater than the molecular mass of 35 833 Da calculated from the reported DNA sequence [Gencic et al. (1991) *Eur. J. Biochem. 199*, 122–131]. The Fe-S protein (subunit V) gives two masses which differ by 60 mass units, presumably due to either the partial loss of the two sulfur atoms or microheterogeneity. Neither mass agrees with the sequence value, the larger mass being 39 mass units lower than expected from the sequence. The molecular masses of subunits VII and X are 81 and 129 Da larger, respectively, than those calculated from their sequences [Borchart et al. (1986) *FEBS Lett. 200*, 81–86; Schägger et al. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 307–311]. Moreover, the HPLC-purified subunit X contains two distinct components: 7298 (Xa) and 7326 Da (Xb), respectively. Initial sequence analysis of the tryptic digests of the two proteins by HPLC/ESI/MS/MS reveals that Xb contains the NH_2-terminal sequence reported by Schägger et al. (1983), while in Xa alanine replaces the NH_2-terminal valine. The data indicate the high precision of electrospray mass spectrometry in determining protein molecular masses and demonstrate its usefulness in verifying protein sequences or determining errors in them.

The cytochrome bc_1 segment of the mitochondrial respiratory chain (complex III, or ubiquinol–cytochrome *c* oxidoreductase, E.C. 1.10.2.2) catalyzes electron transfer from ubiquinol to ferricytochrome *c* and couples this transfer of electrons to the translocation of protons across the inner mitochondrial membrane (Rieske, 1976; Trumpower, 1990). Cytochrome bc_1 is present in mitochondria of all eukaryotic organisms and has been isolated and characterized from a number of different sources. The polypeptide composition of the mammalian cytochrome bc_1 complex, i.e. complex III, has been studied extensively, and it is composed of 11 subunits (Schägger et al., 1986). These subunits are the two core proteins, I and II; the three redox centers, cytochrome *b*

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(subunit III), cytochrome c_1 (subunit IV), and the iron-sulfur protein (subunit V); and six subunits with molecular masses less than 14 kDa. The primary structures of all 11 subunits from the mammalian cytochrome bc_1 complex have been determined either by direct amino acid sequencing analysis (Borchart et al., 1985, 1986; Schägger et al., 1983, 1985, 1987; Wakabayashi et al., 1982a,b, 1985) or by DNA sequence analysis (Anderson et al., 1982; Gencic et al., 1991).

Recently, reverse-phase high-performance liquid chromatography (HPLC)[1] has been successfully used to separate and quantitate the subunit content of bovine cytochrome *c* oxidase (Robinson et al., 1990; Y.-C. Liu, L. Sowdal, and N. C. Robinson, unpublished results). In the present study, a similar approach has been used to develop a facile, rapid procedure for the separation of the subunits of bovine complex III. This C_{18} reverse-phase HPLC procedure is capable of completely resolving 10 of the 11 subunits of the cytochrome bc_1 complex. Because of the high purity of the HPLC-purified subunits, their molecular masses could also be determined by electrospray ionization mass spectrometry (ESI/MS). The ESI/MS molecular masses are of high accuracy, better than ±2 mass units per 10 kDa, and indicate that a number of sequence errors were made in the analysis of many of the cytochrome bc_1 subunits.

EXPERIMENTAL PROCEDURES

Materials

Sodium deoxycholate and sodium cholate were purchased from Sigma Chemical Co. and were recrystallized before use. Dodecyl maltoside was purchased from Anatrace, Inc. Triton X-100 (specially purified) was obtained from Boehringer Mannheim GmbH; Optima grade acetonitrile was from Fisher Chemicals; duroquinone and sodium borohydride were from Sigma Chemical Co.; and HPLC/spectro grade TFA was from Pierce Chemical Co. Electrophoresis grade acrylamide and bis(acrylamide) were purchased from Serva and Kodak, respectively, and were used without further purification. Other chemicals were ACS reagent grade.

Methods

Purification of bc_1 Complex. Cytochrome bc_1 complex was purified from frozen bovine heart as previously described (Musatov & Robinson, 1994). In this method the enzyme was extracted from Keilin–Hartree heart muscle particles with Triton X-100 as described by Schägger et al. (1986), but was purified by ammonium acetate and ammonium sulfate precipitation from deoxycholate and cholate solutions as described by Rieske (1967). After the final ammonium acetate precipitation, the floating layer of red protein was carefully removed, dissolved in Tris–sucrose–histidine buffer (TSH buffer), dialyzed for 2–3 h against TSH buffer, and frozen at −70 °C until used. The cytochrome c_1 content (3.5 ± 0.3 nmol/mg) was determined from the ascorbate-reduced minus ferricyanide-oxidized spectrum using a double difference extinction coefficient of 17.5 mM^{-1} cm^{-1} for 552 minus 540 nm (Yu et al., 1972); the cytochrome *b* content (6.6 ± 0.4 nmol/mg) was determined from the dithionite-reduced minus ascorbate-reduced spectrum using a double difference extinction coefficient of 28.5 mM^{-1} cm^{-1} for 562 minus 576 nm (Berden & Slater, 1970). Protein concentrations were determined by the Biuret method (Gornall et al., 1949). Typical preparations had an enzymatic activity of 180–200 s^{-1}, i.e., nanomoles of cytochrome *c* reduced per nanomole of cytochrome c_1 per second, with duroquinol as a substrate when assayed in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA and 2 mM NaN_3 as described by Nelson and Gellerfors (1978).

Reverse-Phase High-Performance Liquid Chromatography. HPLC analysis of the subunit content of cytochrome bc_1 was performed on a Vydac C_{18} reverse-phase column (5 μm, 0.46 × 25 cm, 300-Å pore size) at 1 mL/min using a Waters/Millipore liquid chromatography system that was controlled by a 486DX2 microcomputer with Waters Millennium 2010 software running under Windows 3.1. Similar results were obtained with a Vydac 10-μm column (0.46 × 25 cm, 300-Å pore size) except that subunits I, II, and XI were not completely resolved. Column effluents were monitored with a Waters 996 UV–vis photodiode array detector, and data were collected, stored, and analyzed on the microcomputer using the above Millennium software. In our system, both an autosampler and a manual injection valve are located between the gradient mixer and the column, which requires that the gradient must flow through a pump dampening coil and both injectors before it reaches the column. We have measured the total volume to be 8 mL between the mixer and column; therefore, attempts to reproduce this work on systems without this large delay will probably result in much earlier elution times for the subunits. To completely reproduce our analysis procedure, an isocratic step of 8 min at 100% solvent A should be added to our protocol prior to starting the acetonitrile gradient.

Sample and Column Preparation. Cytochrome bc_1 (50–700 μg) was prepared for C_{18} reverse-phase HPLC analysis by a protocol that was similar to that used for subunit analysis of cytochrome *c* oxidase (Robinson et al., 1990). With cytochrome bc_1, two minor but important changes had to be made in the procedure: (i) the final concentration of dodecyl maltoside in the sample must be at least 5 mg/mL; (ii) the sample must be incubated at room temperature for at least 45–60 min after acidification to 0.2% TFA.

Injection and Acetonitrile Gradient Elution. The column was equilibrated with water containing 0.2% TFA for at least 30 min prior to injection of a sample. The sample (50–700 μg in water containing 5 mg/mL dodecyl maltoside and 0.2% TFA) was loaded via a Waters autosampler. The subunits were separated with a water/acetonitrile gradient using 0.2% TFA in water (solvent A) and 0.2% TFA in acetonitrile (solvent B). The gradient consisted of a 0–50-min linear gradient from 0 to 50% solvent B (1%/min) and a 50–90-min linear gradient from 50 to 60% solvent B (0.25%/min). Fractions (0.5 mL) were collected for analysis by either SDS–PAGE or ESI/MS. It is important that samples to be analyzed by ESI/MS are collected in polypropylene tubes. Samples were prepared for ESI/MS by evaporation to dryness in a Speed-Vac vacuum centrifuge concentrator, after which they could be stored at 4 °C until analyzed.

Electrospray Ionization Mass Spectrometry. ESI mass spectra were acquired on a Finnigan-MAT SSQ700 quadrupole mass spectrometer fitted with an Analytica of Brandford electrospray source. The electrospray voltage was −3.5 kV. The dried samples of cytochrome bc_1 subunits, obtained from 700 μg of cytochrome bc_1, were dissolved in 15 μL of 5% aqueous acetic acid and mixed with 135 μL of 50% aqueous

[1] Abbreviations: HPLC, high-performance liquid chromatography; ESI/MS, electrospray ionization mass spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; TSH buffer, 20 mM Tris-HCl buffer, pH 8.0, containing 0.66 M sucrose and 0.001 M histidine.

acetonitrile. Samples were then diluted again 1:5 with 50% aqueous acetonitrile, and a few microliters was infused into the electrospray chamber at a flow rate of 1 μL/min; data were acquired by signal averaging over several minutes. Spectral deconvolution was accomplished by means of the BIOMASS program supplied by Finnigan-MAT.

NaDodSO$_4$-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Each HPLC peptide-containing fraction was analyzed for its subunit content by SDS–PAGE using 6-cm-long gels and either the Tricine buffer method of Schägger and von Jagow (1987) or the 15% acrylamide/SDS/2 M urea system described by Robinson et al. (1990). The first method separates all 11 subunits and is particularly good at resolving the low molecular weight subunits, but the latter system gives better separation of the four largest molecular weight subunits. A digitized picture of the Coomassie Blue stained gel was captured using a Philips monochrome CCD camera that was connected to an Apple Macintosh IIsi computer via a Quick Capture frame grabber interface computer board purchased from Data Translation, Inc. Analysis of the captured video image to yield the appropriate densitometric scans was accomplished using version 1.53 of the National Institutes of Health Image software run on a Macintosh IIsi computer.

NH$_2$-Terminal Protein Sequences. Five hundred picomoles of HPLC-purified subunit I in acetonitrile/water/TFA was applied to a Millipore pSQ Immobilon membrane, and the first 14 amino acids were determined by automated Edman degradation with an Applied Biosystems Model 477A gas/liquid phase sequencer by analyzing the resultant PTH-amino acids on a Model 120A HPLC analyzer. Sequential yields were approximately 90% after an initial 50% yield on the first cycle.

RESULTS

Subunit analysis of the cytochrome bc_1 complex by C_{18} reverse-phase HPLC required somewhat different incubation and gradient elution conditions than had previously been successful with bovine cytochrome *c* oxidase (Robinson et al., 1990; Y.-C. Liu, L. Sowdal, and N. C. Robinson, unpublished results). With cytochrome bc_1, two relatively minor but important changes had to be made in the sample preparation and elution protocols: (i) during both sample preparation and gradient elution, the concentration of TFA had to be increased to 0.2%, and (ii) at least 5 mg of dodecyl maltoside per milliliter had to be present during the 45–60-min sample incubation in water containing 0.2% TFA. With these changes, 10 of the 11 subunits of cytochrome bc_1 could be separated with almost baseline resolution on a 5-μm, 300-Å pore, 25 × 0.46 cm C_{18} Vydac column (Figure 1). Identification of the peak containing cytochrome c_1 (subunit IV) is the easiest since it contains a covalently bound heme and can be identified by monitoring the elution absorbance at 416 nm. The other broad peak with absorbance at 416 nm is presumably due to the two protoporphyrin IX's extracted from each cytochrome *b* (subunit III) since the elution peak does not coincide with the elution of any protein and has the spectral properties of protoporphyrin IX. Positive identification of the subunit content in each of the eluting peaks was made by comparing its relative migration on SDS–PAGE with a similar analysis of the entire complex. Several examples of this type of identification are illustrated in Figure 2. Confirmation of the subunit content of several peaks was also made by UV–visible spectroscopy. Subunits VII, VIII, and IX contain no tryptophan (Borchart et al., 1985, 1986; Wakabayashi et al., 1982b); therefore, their identifications were easily confirmed

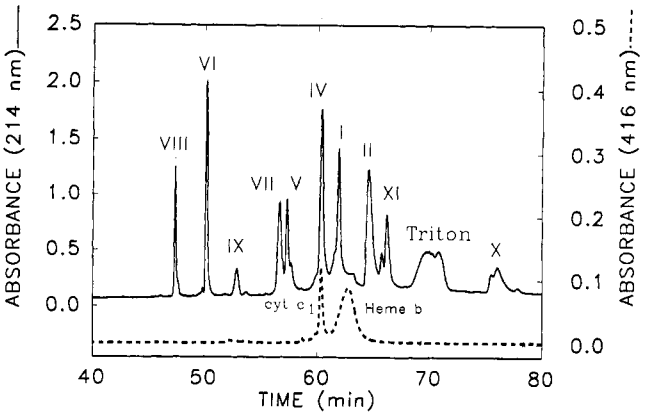

FIGURE 1: Reverse-phase HPLC separation profile of cytochrome bc_1 subunits. Seven hundred micrograms of protein was injected on a 5-μm, 300-Å pore size Vydac C_{18} column (0.46 × 25 cm). Gradient elution of the subunits at 1 mL/min was as described in Experimental Procedures. The eluant was monitored for absorbance at 214 (—) and 416 nm (- - -). The broad peak eluting at about 70 min with absorbance at 214 nm did not contain protein and was due to 0.14 mg of residual Triton X-100 per milligram of cytochrome bc_1 that remained from the isolation procedure.

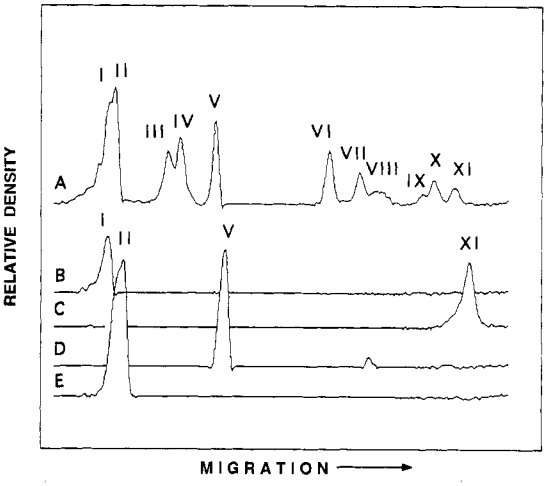

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cytochrome bc_1 subunits. Samples were loaded and separated on a 6.0-cm-long slab gel using the Tricine buffer system of Schägger and von Jagow (1987). Scan A, 6 μg of the entire cytochrome bc_1 complex; scans B, C, D, and E, reverse-phase HPLC-purified subunits I, XI, V, and II, respectively.

by comparison of chromatograms monitored at 214 with those obtained at 280 nm (data not shown). By these approaches identification of the subunit content of each of the HPLC elution peaks with 10 of the 11 subunits of cytochrome bc_1 was possible.

The one subunit which was not eluted from the C_{18} reverse-phase column was cytochrome *b*. This is probably due to the insolubility of cytochrome *b* in more than 25% acetonitrile containing 0.2% TFA. Insolubility was determined by (i) incubation of the cytochrome bc_1 complex in various mixtures of water and acetonitrile with 0.2% TFA, (ii) filtration of the solutions through a Millex HV filter, and (iii) analysis of the filtrates for missing subunits by SDS–PAGE. All other subunits were soluble in 0.2% TFA solutions containing less than 60% acetonitrile. The insolubility of cytochrome *b* in the acidic acetonitrile containing eluate precluded its elution from the reverse-phase column.

The purity of subunits prepared by reverse-phase HPLC is very high, and the acidic acetonitrile/water eluting solvent is ideally suited for ESI/MS. Each of the 10 subunits eluting

Table 1: Molecular Weights of Subunits of Bovine Cytochrome bc_1 Complex

subunit	SDS–PAGE[a,e]	reported mass[b]	calculated mass[c]	ESI/MS mass[d]
I	49 000	35 833[f]	35 836	49 236
II	47 000	46 520[f]	46 523	46 530
III	43 700	42 590[g]	42 590	ND[p]
IV	27 900	27 924[h]	27 923	27 906
V	25 000	21 708[i]	21 707	21 669/21 609
VI	13 400	13 389[j]	13 388	13 389
VII	9500	9507[k]	9506	9587
VIII	9200	9175[l]	9175	9172
IX	8000	7998[m]	7997	7997
X	7200	7189[n]	7197	7298/7326
XI	6400	6520[o]	6520	6520

[a] Molecular weights in reference *e* which are in good agreement with our SDS–PAGE data. [b] Molecular weights reported in the amino acid sequence publications. Values for subunits IV and V include 1 heme C and 2 Fe-2S groups, respectively. [c] Molecular weights calculated with the MacProMass program. [d] Experimentally determined mass by ESI/MS. Double values for subunits V and X reflect the two major components found in each sample. [e] Schägger et al. (1986). [f] Gencic et al. (1991). [g] apoprotein, Anderson et al. (1982). [h] Wakabayashi et al. (1982a). [i] Schägger et al. (1987). [j] Wakabayashi et al. (1985). [k] Borchart et al. (1986). [l] Wakabayashi et al. (1982b). [m] Borchart et al. (1985). [n] Schägger et al. (1983). [o] Terzi et al. (1991). [p] ND, not determined.

from the reverse-phase column was collected in polypropylene tubes, dried, and taken up in acidic acetonitrile/water, and their masses were determined by ESI/MS. Although some subunits were more difficult to analyze than others, we were ultimately successful in analyzing each of the 10 subunits. As illustrated in Table 1, our experimentally obtained molecular weights for subunits II, VI, VIII, IX, and XI are in excellent agreement with values calculated from the reported amino acid or DNA sequences, differing by less than ±2 mass units per 10 000 Da. For example, subunit II, which is composed of 439 amino acids and has a calculated protein molecular weight of 46 523 (Gencic, et al., 1991), was successfully analyzed. A series of charge to mass peaks between 30+ and more than 60+ were detected (Figure 3A) which yielded a single deconvoluted mass spectrum of subunit II with a molecular mass of 46 530 (Figure 3B). With subunit VIII the ESI/MS data clearly demonstrates that our preparations of subunit VIII do not contain the Lys/Arg microheterogeneity at position 36 that was detected by Wakabayashi et al. (1982b), but only contain the Arg isotype.

Mass values for four other subunits, i.e., subunits IV, V, VII, and X, were not as close to the calculated masses, but were within 100 Da. Cytochrome c_1 (subunit IV) had an ESI/MS molecular mass of 27 906 Da, which is 17 mass units lower than the value calculated from the sequence of Wakabayashi et al. (1982a). This difference is most likely due to either an amino acid replacement, e.g., asparagine for phenylalanine, methionine for asparagine, or serine for alanine, or the elimination of H_2O by dehydration during its isolation or preparation for ESI/MS. The difference between the ESI/MS-determined mass and the calculated mass for subunit VII was even larger, the ESI/MS value being 80 mass units larger than expected. The larger value could be due to the presence of a phosphate ester, which would increase the mass by 81 mass units, but at this time it is impossible to rule out the possibility that the difference is due to an amino acid substitution in our preparation of subunit VII, e.g., histidine for glycine.

Two principal components were observed in the fraction containing the iron–sulfur protein (subunit V, or Rieske protein). The deconvolution spectrum of subunit V gave two

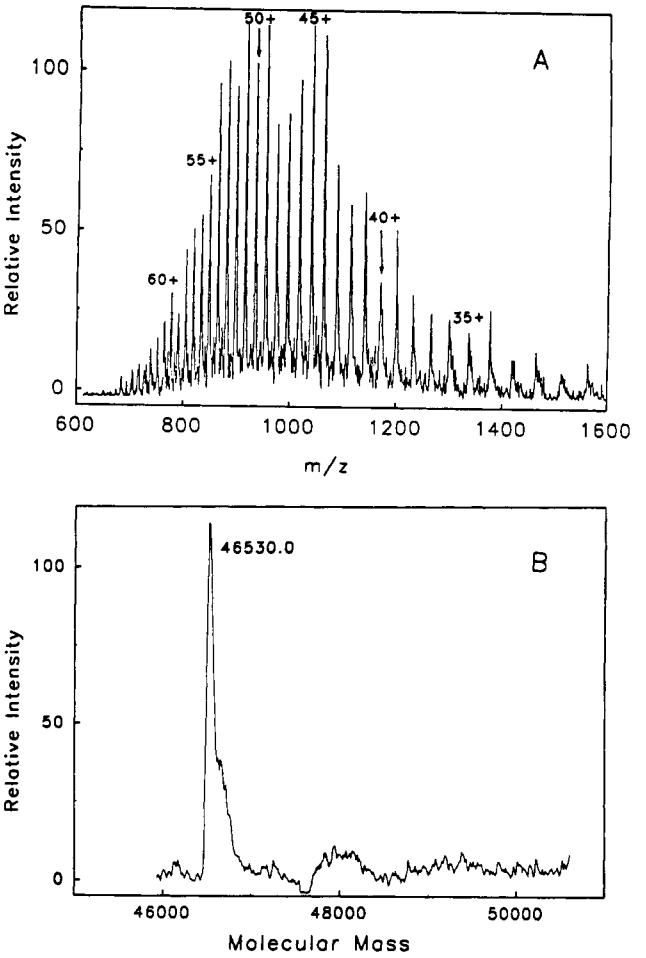

FIGURE 3: Electrospray ionization mass analysis of subunit II. Panel A: The original electrospray ionization mass spectrum. Panel B: The deconvoluted electrospray mass spectrum obtained as described in Experimental Procedures.

masses with a difference of 61.4 ± 2 mass units (Figure 4A) with a range from 59.5 to 64.0 mass units in five different determinations. Most likely this difference is due to a single amino acid replacement, e.g., methionine for alanine. However, it could be caused by the partial loss of the two acid-labile sulfur atoms during the HPLC analysis in 0.2% TFA; during the drying step, which would significantly increase the percentage of TFA; or during the ESI/MS sample preparation in 5% acetic acid. Loss of the two sulfurs would result in a decrease of 64 mass units, which is just within the range of the observed difference. There is still a discrepancy between our observed value of 21 669 Da and the reported value of 21 708 Da which must be due to an amino acid substitution.

Analysis of subunit X caused a few separate problems. First, it is likely that there is a typographical error in the data of Schägger et al. (1983) since they report a molecular weight of 7189, but we calculate a molecular weight of 7197 from their sequence with the MacProMass software. Moreover, two distinct components are present in the deconvolution spectrum of the HPLC-derived subunit X with masses of 7298 (Xa) and 7326 mass units (Xb), neither of which matches the value derived from the sequence (Figure 4B). Two bands were not found upon SDS–PAGE analysis of this fraction, but the HPLC elution peak clearly shows a doublet eluting from the column between 76 and 78 min (Figure 1) which we attribute to the two masses detected by ESI/MS. This was confirmed by ESI/MS analysis of fractions collected across the doublet peak; analysis of the leading side of the doublet showed that it was enriched in subunit Xa, while similar

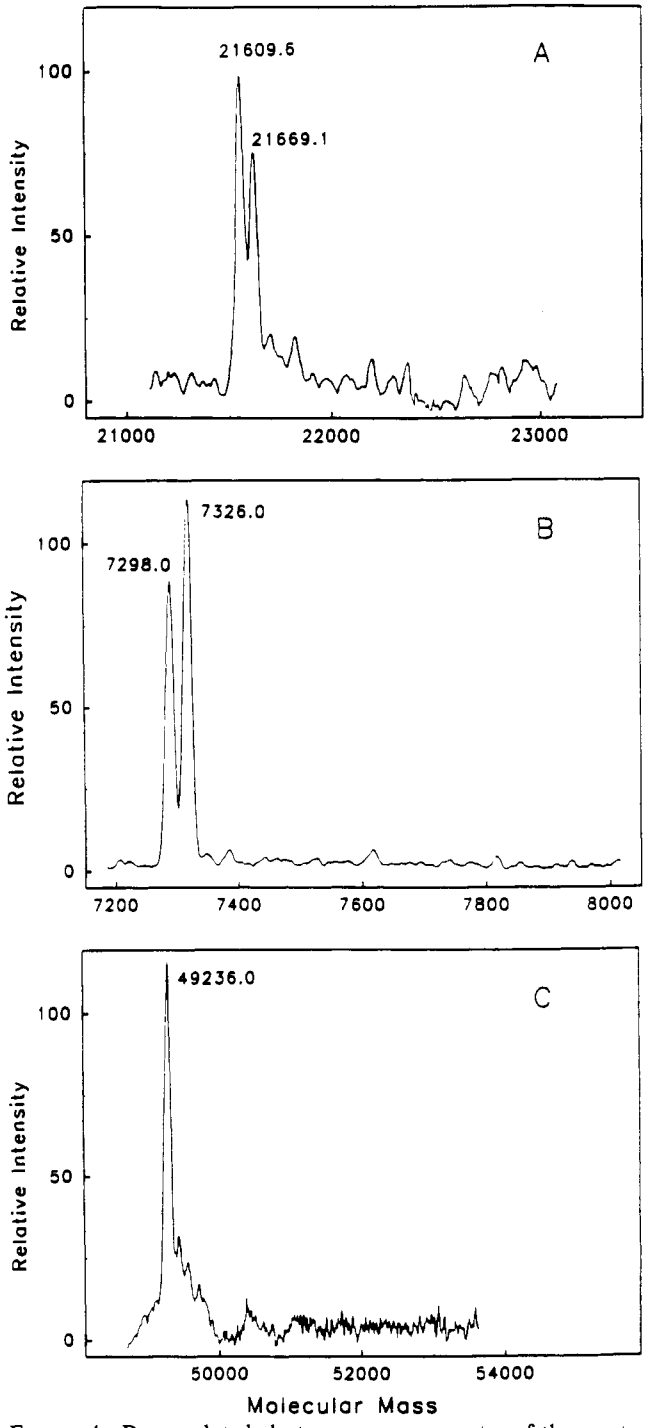

FIGURE 4: Deconvoluted electrospray mass spectra of three cytochrome bc_1 subunits. Panel A: HPLC-purified subunit V. The mass predicted from the sequence of Schägger et al. (1987) is 21 707 mass units. Panel B: HPLC-purified mixture of subunits Xa and Xb. The mass predicted from the sequence of Schägger et al. (1983) is 7197 mass units. Panel C: HPLC-purified subunit I. The mass predicted from the sequence of Gencic et al. (1991) is 35 836 mass units.

analysis of the trailing side showed that it was enriched in subunit Xb. The difference of 28 mass units between Xa and Xb suggested a replacement of alanine for valine. Sequence analysis of the NH_2-terminal tryptic peptides derived from subunits Xa and Xb was done for us by Dr. Donald Hunt using the HPLC/ESI/MS/MS technique. This analysis confirmed that the 7326-Da species (Xb) contains the NH_2-terminal sequence reported by Schägger et al. (1983), while in the 7298 Da species (Xa) alanine replaces the NH_2-terminal valine. Since the mass of Xb is 129 mass units greater than the value calculated from the sequence, it is probable that our subunit Xb contains an additional amino acid, most likely glutamine (128.13), lysine (128.18), glutamic acid (129.12), or methionine (131.2), or that it contains an amino acid substitution, e.g., tryptophan for glycine (129.17).

Although the ESI/MS masses for 9 of the 10 HPLC-derived subunits of cytochrome bc_1 are all within 130 mass units of the calculated values, the ESI/MS mass for subunit I is very different from the mass calculated from the cDNA sequence reported by Gencic et al. (1991). The deconvolution mass spectrum (Figure 4C) clearly indicates a mass of 49 236 rather than 35 836 mass units as calculated from the cDNA sequence; no other major components were found in this fraction. The 13 400 mass unit difference cannot easily be explained even by a major posttranslational modification; however, the ESI/MS value is much more consistent with the SDS–PAGE molecular weight of 47 000–50 000 (Bell & Capaldi, 1976; Mendel-Hartvig & Nelson, 1983; Schägger et al., 1986). We confirmed that the HPLC-derived subunit I was indeed the core I polypeptide by NH_2-terminal sequence analysis. The first 14 amino acids were identical to those reported for the core I polypeptide (Gencic et al., 1991; Capaldi et al., 1988).

DISCUSSION

Subunit analysis of each of the mitochondrial electron-transport complexes has traditionally been done by SDS–PAGE. However, each of these multi-subunit complexes contains half a dozen or more subunits smaller than 15 kDa, many of which are quite difficult to resolve by even the best SDS–PAGE methods. In fact, as new SDS–PAGE methods have been developed with increased resolving power for small proteins, the number of subunits believed to be part of each of these complexes has almost doubled.

To complement the powerful technique of SDS–PAGE, we have developed a reverse-phase HPLC system for analyzing the subunit content of these complexes. The advantage of the reverse-phase HPLC approach is that it separates polypeptides on the basis of their hydrophobicity, not their molecular weight, so that two subunits that are difficult to separate on SDS–PAGE often are very easy to separate by reverse-phase HPLC. Previously, this approach has been used to separate all 10 of the nuclearly encoded subunits of cytochrome *c* oxidase (Robinson et al., 1990; Y.-C. Liu, L. Sowdal, and N. C. Robinson, unpublished results). With quite minor modifications, the same approach has now been used to separate the 10 nuclearly encoded subunits of the cytochrome bc_1 complex (refer to Figure 1). Only the mitochondrially synthesized and apolar subunit III, i.e., cytochrome *b*, a subunit that is rather insoluble in acid acetonitrile/water, was not eluted from the C_{18} column. Subunits that are quite difficult to separate by SDS–PAGE, e.g., IX, X, and XI, are very well resolved by reverse-phase HPLC, eluting at 53, 77, and 66 min, respectively (refer to Figure 1). In fact, the resolving power is so good that unknown microheterogeneity in subunit X could be detected by the doublet peak eluting from the HPLC column (refer to Figure 1). In contrast to cytochrome *c* oxidase in which the largest subunits did not elute from the column, even the two 50-kDa core proteins were obtained in reasonably good yield.

Reverse-phase HPLC is a rapid preparative method, and the eluant can be analyzed on-line or post column by a variety of techniques. Ten of the 11 subunits of bovine cytochrome bc_1 can be isolated in less than 3 h, including the sample preparation time. Multiwavelength monitoring of the eluant not only permits the immediate detection of some subunits, e.g., the heme-containing cytochrome c_1 (subunit IV) or the

tryptophan-lacking subunits VII, VIII, and IX, but also permits the quantitative determination of the amount of covalently or non-covalently bound chromophores. For example, in the present study, our preparations of cytochrome bc_1 still contained 0.14 g of Triton X-100 per gram of protein (caption to Figure 1) even though the enzyme had been precipitated several times from both deoxycholate and cholate solutions after its exposure to Triton X-100. Similarly, by detecting shifts in elution peak positions or by monitoring the elution of fluorescence, multiwavelength detection of the reverse-phase HPLC eluant can be used to quantify and determine chemical labeling of specific subunits by fluorescent and/or absorbing chromophores (Y.-C. Liu, L. Sowdal, and N. C. Robinson, unpublished results). Because reverse-phase HPLC is a preparative technique, we were able to use this to advantage in the present study for the isolation of each of the subunits in highly pure form for subsequent analysis by ESI/MS.

The ESI/MS technique is presently unsurpassed in its accuracy for the determination of whole protein molecular weights. In the present analysis, molecular masses were determined to better than ±2 mass units per 10 kDa. Even the quite large subunit II, which has a molecular weight of 46 523, was analyzed to an accuracy of 7 mass units as compared with the sequence-derived molecular weight. Because the values obtained for subunits II, VI, VIII, IX, and XI are all within this degree of accuracy, we conclude that the ESI/MS results are in complete agreement with the protein- and/or cDNA-derived sequences for these subunits. However, the ESI/MS data that were obtained for the other five subunits are significantly different from the sequence-derived molecular weights.

The ESI/MS data obtained for subunit I, i.e., core protein I, is by far the most interesting. The molecular weight of 49 236 for subunit I is clearly far larger that the value of 35 836 calculated for the cDNA sequence of Gencic et al. (1991). We believe that the ESI/MS value for the molecular weight of subunit I is correct and that the cDNA-derived amino acid sequence of Gencic et al. (1991) must be missing a carboxy-terminal sequence of 13 400. This conclusion is based upon the following evidence: (1) we did measure the molecular mass of the same protein subunit that Gencic et al. (1991) sequenced since the first 14 amino acids of the NH_2 terminus of our subunit I were identical to the NH_2-terminal sequence published by Gencic et al. (1991) and by Capaldi et al. (1988); (2) the ESI/MS data is much closer in agreement with the SDS–PAGE-derived molecular weight of 47 000–50 000 for subunit I (Bell & Capaldi, 1976; Mendel-Hartvig & Nelson, 1983; Schägger et al., 1986); (3) the recently published cDNA sequence for the human type subunit I (Islam et al., 1994) predicts a protein sequence that is 85% homologous with the bovine sequence predicted by Gencic et al. (1991), but it has an additional 122 amino acids at the carboxy terminus. A cloning or sequence error must have occurred during the analysis of the cDNA for bovine subunit I which predicted an early termination codon.

The ESI/MS molecular masses of four other subunits also did not agree with the published sequence data, i.e., cytochrome c_1 (subunit IV) and subunits V, VII, and X. The value for cytochrome c_1 is only 17 mass units lower than the sequence-predicted molecular weight of 27 923 and is probably due to some experimental artifact, e.g., dehydration. As discussed in the Results, the two mass values obtained for the Rieske iron–sulfur protein (subunit V) could be due to either microheterogeneity or the partial loss of the two acid-labile sulfurs during the acidic treatment of the subunit while it was purified by HPLC or prepared for ESI/MS. However, neither molecular mass matches the published sequence data. The difference of 38 mass units could represent a replacement of one of the histidines in the reported sequence by valine (a difference of 38) or replacement of one of the tryptophans with phenylalanine (a difference of 39). The data for subunit VII are also inconsistent with the molecular weight calculated for the sequence, the ESI/MS value being 80 ± 2 mass units too large. Once again, the larger ESI/MS value may suggest an amino acid replacement, but more likely it suggests the presence of a phosphate ester, which would increase the mass by 81.

The data for subunit X are also unusual and quite interesting. Both the ESI/MS data and the doublet observed in the HPLC elution profile suggest two isoforms of subunit X, i.e., subunit Xa and Xb. The difference in the two subunits is caused by the presence of an NH_2-terminal alanine in subunit Xa rather than an NH_2-terminal valine in subunit Xb. Other than this NH_2-terminal substitution, the isoforms are identical. This type of microheterogeneity most likely is the result of two gene products. However, the mass of subunit Xb, which has the NH_2-terminal valine in agreement with the sequence of Schägger et al. (1983), is still 129 mass units larger than that derived from the sequence and suggests that our subunit Xb contains an additional amino acid, most likely glutamine (128.13), lysine (128.18), glutamic acid (129.12), or methionine (131.2).

The combination of the high resolving power of reverse-phase HPLC with the precise mass determination of molecular masses by ESI/MS is a powerful approach to characterizing a multi-subunit membrane complex like cytochrome bc_1. By this approach we have verified five of the subunit sequences and also clearly demonstrated subunit microheterogeneity in subunit X, probable amino acid substitutions in subunit V, a possible phosphorylation in subunit VII, an additional amino acid that should be included in the sequence of subunit Xb, and the large carboxy-terminal sequence of more than 13 kDa that is missing in subunit I. Because the method is relatively simple and highly accurate and has been applied to at least two multi-subunit membrane complexes, i.e., cytochrome bc_1 and cytochrome *c* oxidase (Y.-C. Liu, L. Sowdal, and N. C. Robinson, unpublished results), it should be considered as a general approach for verifying sequence work on other complexes.

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