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The Study of Bovine Cytochrome c Oxidase Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

presented by

Qian Li

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Department of Chemistry

John Allison

Major Professor's Signature

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Date

THE STUDY OF BOVINE CYTOCHROME C OXIDASE USING MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

By

Qian Li

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ABSTRACT

THE STUDY OF BOVINE CYTOCHROME C OXIDASE USING MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

By

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Noncovalent interactions bind biological complexes such as drug-peptide complexes, duplex DNA, and multi-subunit proteins. A recent analytical tool employed by biochemists to study noncovalent complexes is mass spectrometry. To explore better conditions to stabilize noncovalently bound complexes in the matrix-assisted laser desorption/ionization (MALDI) experiment, the protein cytochrome c oxidase (CcO) was chosen for this study. CcO is a membrane protein. It contains 13 subunits, with a monomer molecular weight over 200,000. CcO provided two challenges in its MALDI MS analysis. The first challenge is related to the fact that it is a membrane protein; these tend to aggregate in water. Subunits I, II, and III are very hydrophobic and not detected in the conventional MALDI experiment. The detergent lauryl maltoside was investigated as an additive to solubilize these hydrophobic subunits. The effects of the concentration of lauryl maltoside and protein on the resulting spectra were investigated. The second challenge is related to the fact that all of intact enzyme completely dissociates in the conventional MALDI MS experiment. Can the intact enzyme be preserved and detected? Our experimental results suggest that with the appropriate matrix, solvents, pH and detergent, the CcO complex can be detected in MALDI MS.

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LIST OF ABBREVIATIONS

ACN	Acetone nitrile
ATT	6-aza-2-thiothymine
BO	Bacterioopsin
BSA	Bovine serum albumin
CcO	Cytochrome c oxidase
CHCA	α-cyano-4-hydroxycinnamic acid
CL	Cardiolipin
CMC	Critical micelle concentration
DE	Delayed extraction
DHB	2,5-dihydroxybenzoic acid
ESI	Electrospray ionization
FTMS	Fourier transform mass spectrometry
GDP	Guanosine diphosphate
GPC	Gel permeation chromatography
GTP	Guanosine triphophate
HABA	2-(4-hydroxyphenylazo (benzoic acid)
HNE	4-hydroxy-2-nonenal
HPLC	High performance liquid chromatography
IR	Infrared
KE	Kinetic energy
LDI	Laser desoption/ionization
LM	Lauryl maltoside

MALDI	Matrix-assisted laser desorption/ionization
[M+H]	Protonated molecule
m/z	Mass-to-charge ratio value
N-terminus	Amino-terminus of peptide
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamines
PG	Phosphatidyl glycerols
PSD	Post-source decay
SA	Sinapinic acid
SDS-PAGE	Sulfate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
TOF	Time-of-flight
UV	Ultraviolet

Chapter One: The Study of Non-covalent Complexes Using MALDI-MS

Introduction

Non-covalent complexes play a very important role in biological system. Proteins serve specific functions and may be transported to their destination by associating with a specific partner. They can interact with other proteins, peptides, oligonucleotides, metal ions and other small molecules. They frequently bind by non-covalent forces. Non-covalent interactions are found everywhere in life science. For example, drugs function by binding to a specific target, most often, proteins or oligonucleotides. A better understanding of their structure will assist researchers in the discovery of small molecules that act as inhibitors to diseases.

Non-covalent forces include: hydrogen bonds, Van der Waals forces, ion-ion interactions and hydrophobic interactions. They are usually much weaker than covalent bonds. A protein structure can be broken down into four levels. The primary structure refers to the amino acid sequence. The secondary structure involves the interaction of amino acids within the chain to form hydrogen-bonded structures such as α-helices and β-sheets. Tertiary structure is the "global" folding of a single polypeptide chain. The major driving force is the hydrophobic effect. Quaternary structure refers to the association of two or more polypeptide chains into a multi-subunit structure. All four types of non-covalent interactions are found in the interactions between subunits in a multi-subunit protein. Except for the primary structure, non-covalent forces are involved in all the higher order structures. They are critical to preserve protein stability and activity. Disruption of these

forces by a change of pH, solvent composition, temperature, ionic strength and addition of reducing reagents may cause partial or complete denaturation of the protein.

Many conventional analytical techniques have been employed to study non-covalent compounds. Centrifugation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography (GPC) have been used, but don't provide enough information regarding molecular weight and stoichiometry of the protein. X-ray crystallography and NMR spectrometry have been used to solve 3-D structures of various proteins, but they lack the direct information for the non-covalent complexes based on molecular weight information [1]. With the development of soft ionization techniques yielding little or no fragmentation such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), mass spectrometry has become a very important method to study non-covalent complexes. They have the obvious advantages of providing accurate molecular and stoichiometric information with great speed and sensitivity.

Why do non-covalent complexes dissociate in MALDI-MS?

MALDI has been demonstrated to be an important method for the structural characterization of proteins. Coupled with the time-of-flight mass analyzer, MALDI has been used to determine molecular weights up to 500,000 Da. It is extremely sensitive. The amount of sample required for MALDI-MS is in the sub-picomole- and quite often even in the low femtomole- range. The sensitivity achieved in MALDI is 10 times that of ESI [1]. Although MALDI is usually better than ESI for detecting high molecular weight

species [2], most of the non-covalent complexes analyzed using mass spectrometry were reported by ESI. They include multi-subunit proteins, protein-nucleic acid, peptide-drug, duplex DNA-drug, polypeptide-metal ion, and protein/peptide-peptide complexes [3, 16]. As shown in Figure 1.1, most non-covalent complexes dissociate when they are analyzed by MALDI-MS.

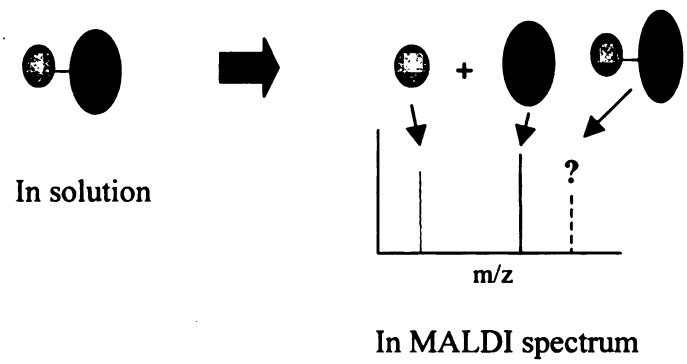

Figure 1.1: Non-covalent compound dissociates in MALDI

The application of MALDI-MS to non-covalently bound compounds may be limited by its harsh sample preparation conditions. In a conventional MALDI experiment, the analyte is usually mixed with matrix in a solvent consisting of water and an organic co-solvent. An acid such as trifluoroacetic acid and formic acid is often used to improve the solubility. After that, the matrix and analyte are cocrystallized on a metal surface. To preserve the native conformation of proteins, physiological conditions should be approached. The low pH and organic solvents used in the MALDI experiment could destroy the protein native structure. The protein inclusion into the matrix crystals could

also break the non-covalent bonds. What's more, the contact with the metal surface is a possibility suggested by some biochemists.

The effect of pH

pH is very important to control the state of multi-subunit proteins. Most proteins at the physiological pH are above their isoelectric pH and have a net negative charge. When the pH is adjusted to their isoelectric point, their net charge will be zero. Charge repulsion of similar molecules will be minimized and many proteins precipitate. When the pH is far below (above) their isoelectric point, the proteins will have a net positive (negative) charge. The like charges repel each other and prevent the protein from aggregating. But in some area where the density of positive (negative) charge is very high, the repulsion forces will be great enough to unfold the protein. That can lead to denaturation.

The pH effect was investigated by several groups. One of the important non-covalent complexes in cancer research is the association of the *ras* protein with either guanosine diphosphate (GDP) or guanosine triphosphate (GTP). Studies of these noncovalent interactions are the basis for the development of the strategy to discover inhibitors for relevant disease processes. *Ras*-GDP was used as a non-covalent model studied using ESI by Ganguly *et al.* [1]. When the pH was adjusted to 4.7, an ESI spectrum was obtained. The deconvolution of this spectrum yielded an average molecular mass of 19,295, which is in good agreement with the molecular weight of the *Ras*-GDP complex. The spectrum is shown in Figure 1.2. When the pH is decreased to 3, there are two sets

of ion distributions in the spectrum. One set corresponds to the intact complex with an average weight of 19, 294. The other set deconvoluted to an average molecular weight of 18, 852, which represents the protonated free apo-*ras* protein. The spectrum is shown in Figure 1.3. When the pH further decreased to 2.7, only the free apo-*Ras* protein was observed. It indicates that, at low pH, the *Ras*-GDP complex unfolded and the free protein was released. The spectrum is shown in Figure 1.4.

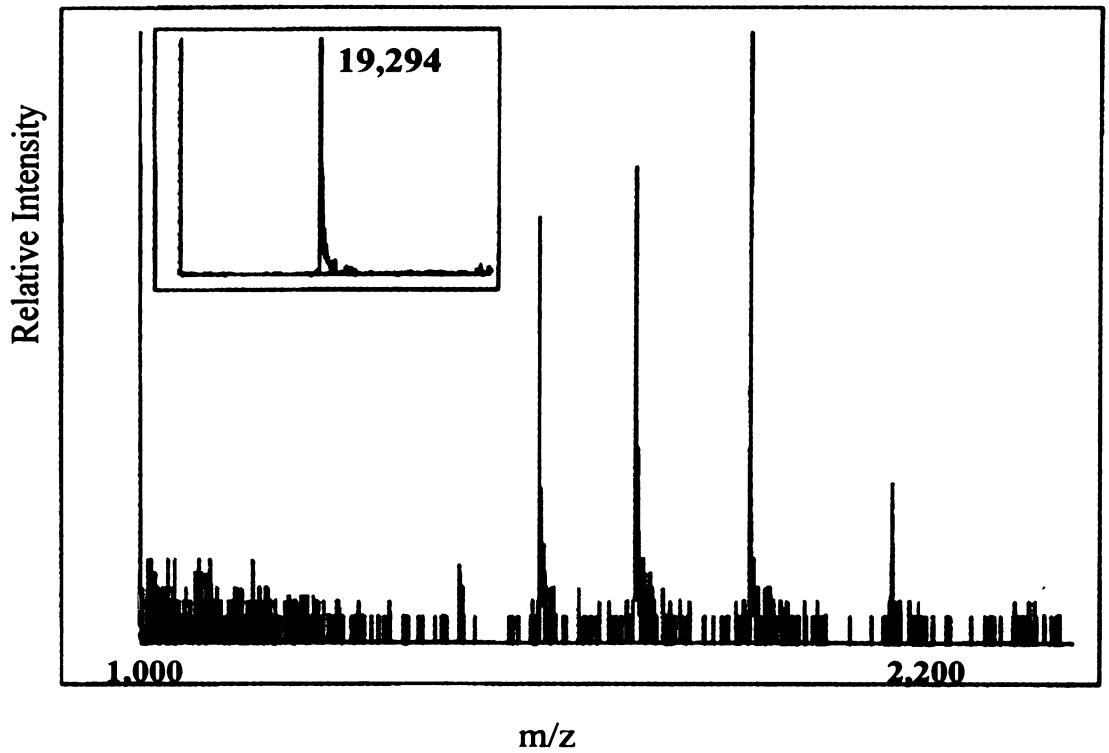

Figure 1.2: ESI mass spectrum of the ras-GDP complex at pH 4.7 in 2 mM ammonium acetate buffer. Inset shows the deconvoluted mass spectrum of the ras–GDP complex with molecular mass 19,294 Da.

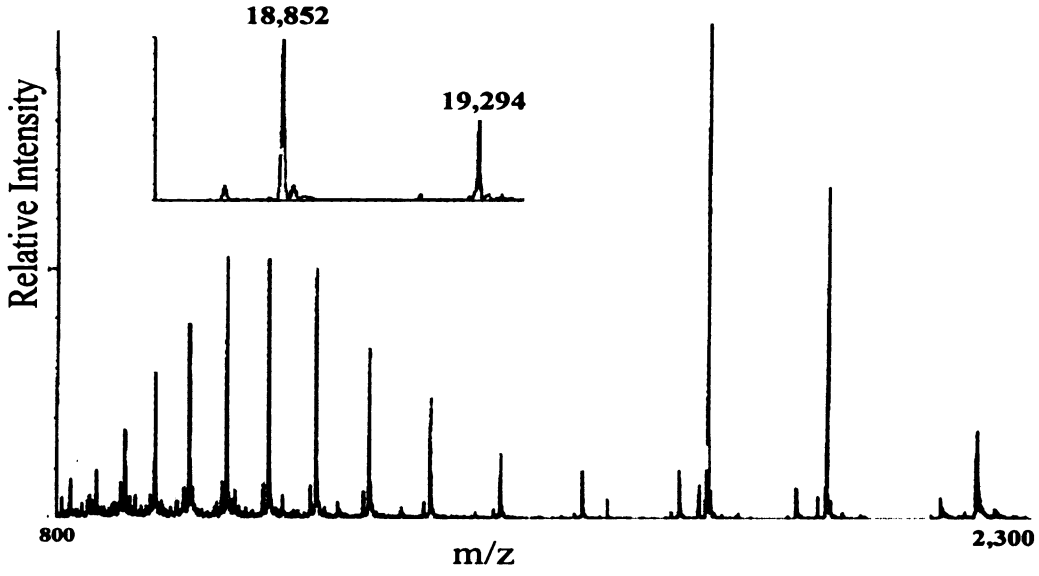

Figure 1.3: ESI mass spectrum of the ras–GDP complex obtained at pH 3 in 2 mM ammonium acetate buffer. Inset shows the deconvoluted mass spectrum of the ras–GDP complex with molecular mass 19,924.4 Da and apo-ras with molecular mass 18,851.4 Da.

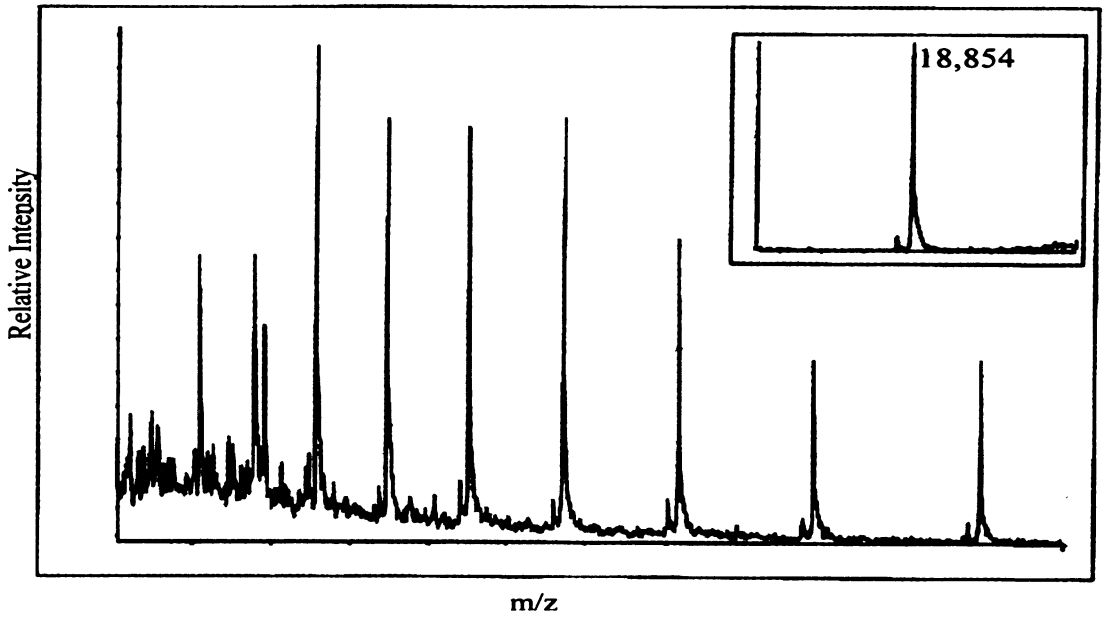

Figure 1.4: ESI mass spectrum of ras–GDP obtained at pH 2.5 in 2 mM ammonium acetate buffer using 20 mM protein solution. Inset shows the deconvoluted mass spectrum of the apo-ras with molecular mass 18, 854 Da.

The effect of pH was also shown in the study of a substrate-enzyme complex by Karas *et al.* [5]. Sinapinic acid was used as matrix. When the matrix was prepared in ethanol-water (1:1) (pH<2), ions for the aminopeptidase-bovine growth hormone-releasing factor (GHRF) were absent from the spectrum. When 1 M ammonium citrate was added to control pH, ions representing the enzyme, substrate and enzyme-substrate complex were observed.

In MALDI-MS, most oligomers dissociate. Human farnesyl protein transferase is a heterodimer consisting of one α- and one β-subunit. MALDI analysis using sinapinic acid dissolved in ACN-0.1%TFA (70:30, v:v) gave peaks corresponding to each of these two subunit monomers. Using sinapinic acid dissolved in ACN-0.2 M Bis-Tris (30:70) (pH=7), a peak representing the heterodimer was detected [6].

All of the above experimental results suggest that pH does play a role in breaking non-covalent bonds. A general way, employed in MALDI MS experiments, to maintain a proper pH for the stabilization of the protein structure is through the use of buffers. Even though MALDI has the advantage of a relatively higher tolerance for impurities such as buffers, detergents and other salts than ESI [8], analysis might be impaired by the interference of the impurities. The analyte must be prepared in a buffer that is not detrimental to MALDI MS analysis. It was found that some buffers alter crystal growth and the analyte signal was not observed [15]. The effect of a variety of buffer solutions on the MALDI-MS experiment has been investigated [7, 15]. The major impact of buffers is the protein incorporation into the matrix crystals and the ionization process. As

the solvent evaporates, there will be a competition among all the components for the matrix crystal surface. Those that are in direct contact with matrix crystals are more easily ionized. The volatility and hydrophobicity are important properties that influence the degree to which the protein is incorporated into the relatively hydrophobic matrix crystals. Ammonium salts are the most commonly used buffers, such as ammonium acetate, ammonium citrate and ammonium bicarbonate. They can be tolerated by MALDI MS analysis at relatively high concentrations (500 mM for ammonium acetate) without degrading the spectrum. They are even found to enhance the signal intensity in some cases [8]. It is not yet clear if the effect is based on the suppression of alkali-adduct formation, the degree of analyte inclusion into the matrix, or a more effective desorption/ ionization process. Farmer and Caprioli [6] studied the specific non-covalent interactions of RNAse S and of leucine zipper dimers. Dissolving both matrix and complex in ammonium hydrogen carbonate, ammonium acetate, and ammonium citrate gave the most intense signals for the complexes.

To overcome the disrupting effect of low pH, the use of a neutral or basic matrix can be another solution. Many basic matrices have been discovered for this purpose [18, 19]. Of particular interest has been the recent observation of several types of non-covalent complexes using 6-aza-thiothymine (ATT) as the matrix. It seems that ATT is becoming a "magic" matrix for non-covalent complex study. The first success was reported on double-stranded oligonucleotides [17]. It has also found success in the analysis of proteins and peptides. Leucine zipper polypeptide dimers were observed in MALDI MS using ATT in buffer solutions whereas the use of CHCA in ACN-0.1%TFA showed no

dimer peaks [6]. Woods *et al.* [9] and Glocker *et al.* [10] also reported the successful use of ATT and ammonium salt buffers to detect zinc finger peptides and enzyme-substrate complexes.

The effect of organic solvents

Solvent composition is another important factor that could lead to the dissociation of non-covalent complexes. The presence of nonpolar organic solvents can weaken the hydrophobic bonds of the proteins and thus the protein structure will be changed.

The disturbing effect of organic solvents was investigated by Ganguly *et al.* using the *Ras*-GDP complex [4]. Methanol was added to a *Ras*-GDP solution at pH 5.8 (this pH can preserve the native conformation) to give a 20% methanol solution. The dissociation of the *Ras*-GDP complex occurred. Both the intact *Ras*-GDP complex and the free apo-*Ras* protein were observed. When the methanol was increased to 50%, the complex could no longer be detected.

Karas *et al.* determined the number of subunits of a multi-subunit complex using MALDI-MS [5]. Streptavidin was mixed with nicotinic acid matrix in 10% ethanol in water. The base peak is the monomer with additional peaks recorded for dimer, trimer, and tetramer. When 50% ethanol was added, only the monomer peak showed up in the spectrum.

Other factors that can disrupt non-covalent interactions

All of the above experimental results suggest that the organic solvent and low pH are the main reasons that limited MALDI to non-covalent complex study, rather than the desorption/ionization process itself. But other factors cannot be completely excluded.

The interaction between matrix and analyte molecules could disrupt the non-covalent bonds. Figure 1.5 shows the non-covalent complex dissociating when incorporating into matrix crystals. The mechanism of how the matrix and analyte cocrystallize is still under investigation. Trial of a variety of matrices would be a solution if some matrices do not disturb the non-covalent bonds.

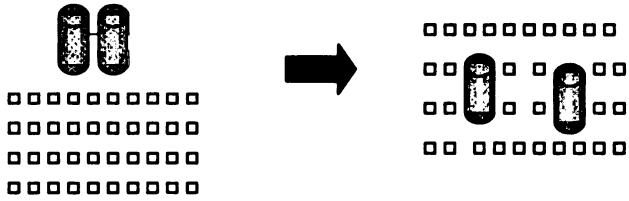

Figure 1.5: Non-covalent complex dissociates when incorporating into matrix crystals

Some biochemists suggested that contact with a metal surface could dissociate non-covalent complexes. The metal surfaces used in MALDI MS are usually gold and steel. If this is true, "sandwich" preparation methods might be useful [11]. In this experiment, a droplet of matrix solution is applied to the sample well first. The solution is dried immediately and a thin layer of matrix crystals is formed. Subsequently, a droplet of a mixture of analyte and matrix is deposited on the thin layer. This avoids the direct

contact of analyte with the metal surface. Moniatte *et al.* reported that, using a sandwich preparation, the crystallization was more homogeneous and more reproducible peaks were obtained [12].

The dissociation of non-covalent compounds could also occur during the desorption and ionization process as shown in Figure 1.6. The bonds may be too weak to survive the laser irradiation. Crosslinking reagents can be used to stabilize the bonding. Farmer *et al.* demonstrated that glutaraldehyde is a useful crosslinking chemical to join protein subunits covalently [6, 13]. Glutaraldehyde reacts primarily with the ε-amino group of lysine to form cross-linking chains of differing lengths. The protein glucose-6-phosphate dehydrognase is known to exist as a homodimer in solution. The dimer signal was increased substantially after the incubation with glutaraldehyde.

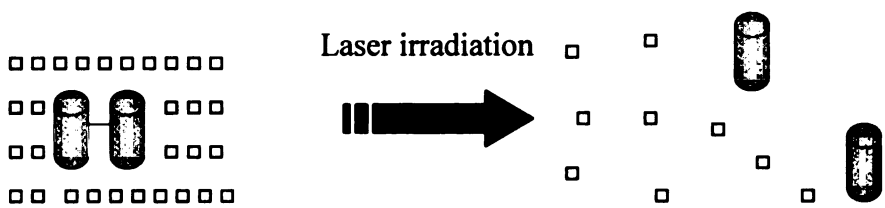

Figure 1.6: Non-covalent complex dissociation could occur in the desorption/ionization process

Chemical additives can be added to stabilize the non-covalent structure. Spermine and methylene blue are found to bind to small DNA duplexes and stabilize them [20]. Glycerol is reported to stabilize proteins in ESI MS [21].

"First shot" phenomenon

A "first shot" phenomenon was observed in many cases in the study of non-covalent complexes using MALDI-MS [6, 12, 14]. That is, the complex peak was detected by the accumulation of the first few laser shots on a specific location. Subsequent laser shots onto this location resulted in the failure to detect non-covalent species. Figure 1.7 a) shows the spectrum of "first shots" of porin, consisting of three identical subunits, each with a molecular weight of about 37 kDa. Subsequent exposure of the same sample area yielded only ions of subunits as shown in Figure 1.7 b). Two reasons were addressed to explain the "first shot" phenomenon. A small amount of protein may precipitate onto the surface of the matrix crystal and then is desorbed as an intact complex by the first few laser shots. Those that were included into the matrix crystals dissociated into their individual parts when they interact with the matrix molecules. Another explanation is: the first shot of the laser on the sample area will result in photodissociation of the protein. As a consequence, subsequent laser shots onto the same spot resulted in the detection of the dissociated parts. The latter hypothesis was ruled out by an irradiation experiment. The sample was irradiated by a UV lamp prior to the MALDI MS analysis simulating UV exposure in MALDI desorption. MALDI MS analysis of these irradiated samples gave results identical to the non-irradiated samples. So they concluded that the intact complex was only maintained at the surface.

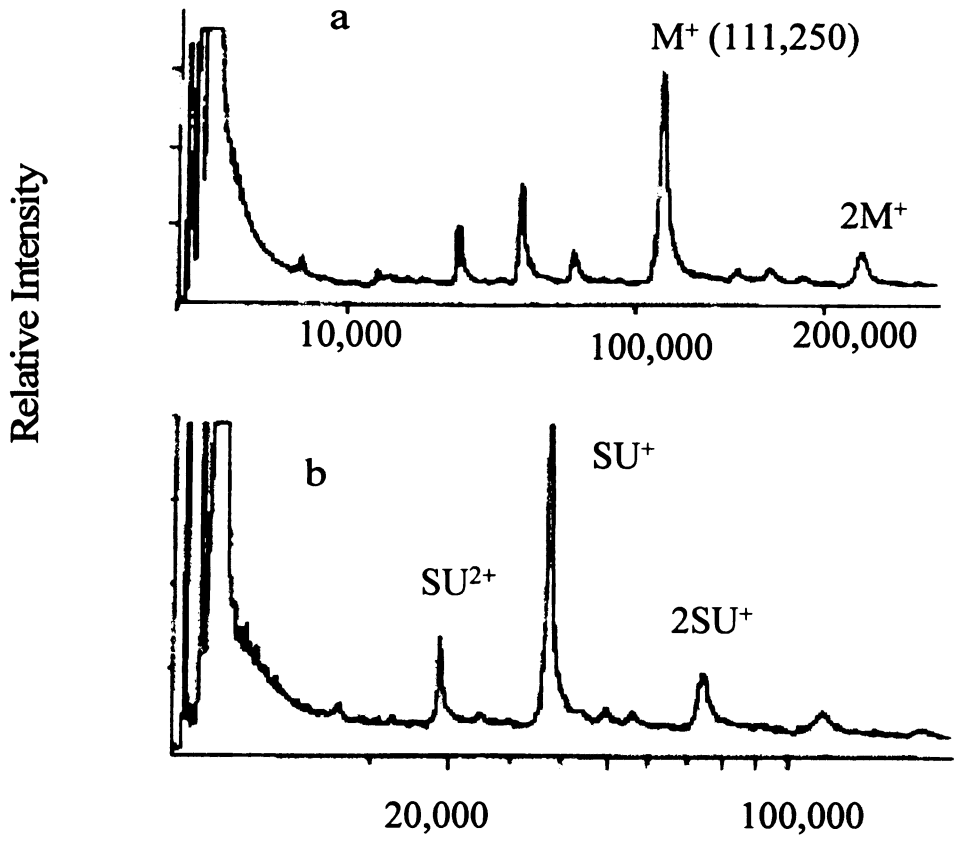

Figure 1.7: a) Sum of first shots on a given spot.
b) Sum of second and following shots on a given spot

Could observed complexes in MALDI MS come from non-specific aggregation?

Non-specific aggregation of binding partners in solution or in the gas phase is a possibility we should be aware of. That is, the complex detected might come from random association rather than specific bonding. Dilution experiments should be performed to eliminate the possibility of non-specific condensation. Aerolysin is a virulence factor secreted by human pathogen *Ad hydrophola.* After activation and

concentration, this protein condenses to form a transmembrane channel responsible for target cell death. The oligomer was found to be a heptamer. MALDI-TOF-MS was used to confirm the stoichiometry of the complex [12]. In the spectrum, except for the heptamer, dimer, trimer and tetramer with decreasing intensities were also observed. They might indicate a non-specific condensation formed in the sample preparation or in the gas phase. In dilution studies, the trimer and tetramer were detected with the same intensity ratio when the sample was diluted or concentrated five times. A non-specific aggregation would at lease produce a small variation in the signal intensity ratio or a small amount of pentamer and hexamer that wasn't observed. This suggests that the formation of trimer and tetramer was due to specific non-covalent interactions.

In this thesis, MALDI-MS will be used to study a multi-subunit protein, bovine cytochrome c oxidase, which consists of thirteen different subunits. It will be shown that pH and organic solvents are the primary limitations to the detection of the complex.

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Chapter Two: Introduction to Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

Introduction

Since its introduction in 1988, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been established as an important analytical instrument for biochemical and biomedical research. The successful use of a matrix extended the application of original laser desorption MS to large molecules [1]. It has many advantages over other ionization methods. Table 2.1 compares different ionization methods of MS for the analysis of proteins and peptides.

It was reported that MALDI-MS can be used to determine molecular weights up to 1,500,000 [2]. It has the advantage of high accuracy and short analysis time. It has been successfully used in the analysis of proteins, oligonucleotides, and biopolymers [3].

Table 2.1: Ionization Methods for Analysis of Proteins and Peptides by MS [29]

Ionization method	For MW information upper mass limit (Da)	Advantages	Disadvantages
Fast atom bombardment	20,000	Ability to analyze mixtures; MW and limited sequence information for pure small peptides; adaptable for LC/MS; widely available.	Matrix interference below m/z 500; widely variable response; peptide-induced suppression effects; limited to flow rates of ≤10 μL/min.
Electrospray ionization	≥150,000	Ability to analyze mixtures; limited sequences information for pure small peptides; adapts easily for LC/MS; most suitable for quadruple MS.	Multiple charging may complicate interpretation of data; glycoproteins may not yield useful information.
Matrix-assisted laser desorption/ionization	>250,000	Highest achieved mass range for proteins and glycoproteins; relatively insensitive to salts; ability to analyze mixtures; simple to operate.	Low resolution; ability to detect structural variants; not presently adapted for LC/MS; limited structural information.
Plasma desorption	≤50,000	Simple to operate; ability to analyze mixtures; limited sequence information for pure small peptides.	Low resolution; ability to detect structural variants; not presently adapted for LC/MS; long acquisition time, often >1h.

Instrumentation

The linear MALDI-TOF-MS instrument diagram is shown in Figure 2.1. Ions are formed in the MALDI source. A N_2 laser with a wavelength of 337 nm (pulsed width of 3 ns) is

used. Ions are accelerated by an electric field in the ion source. They then drift down the flight tube and hit the front plate of the detector.

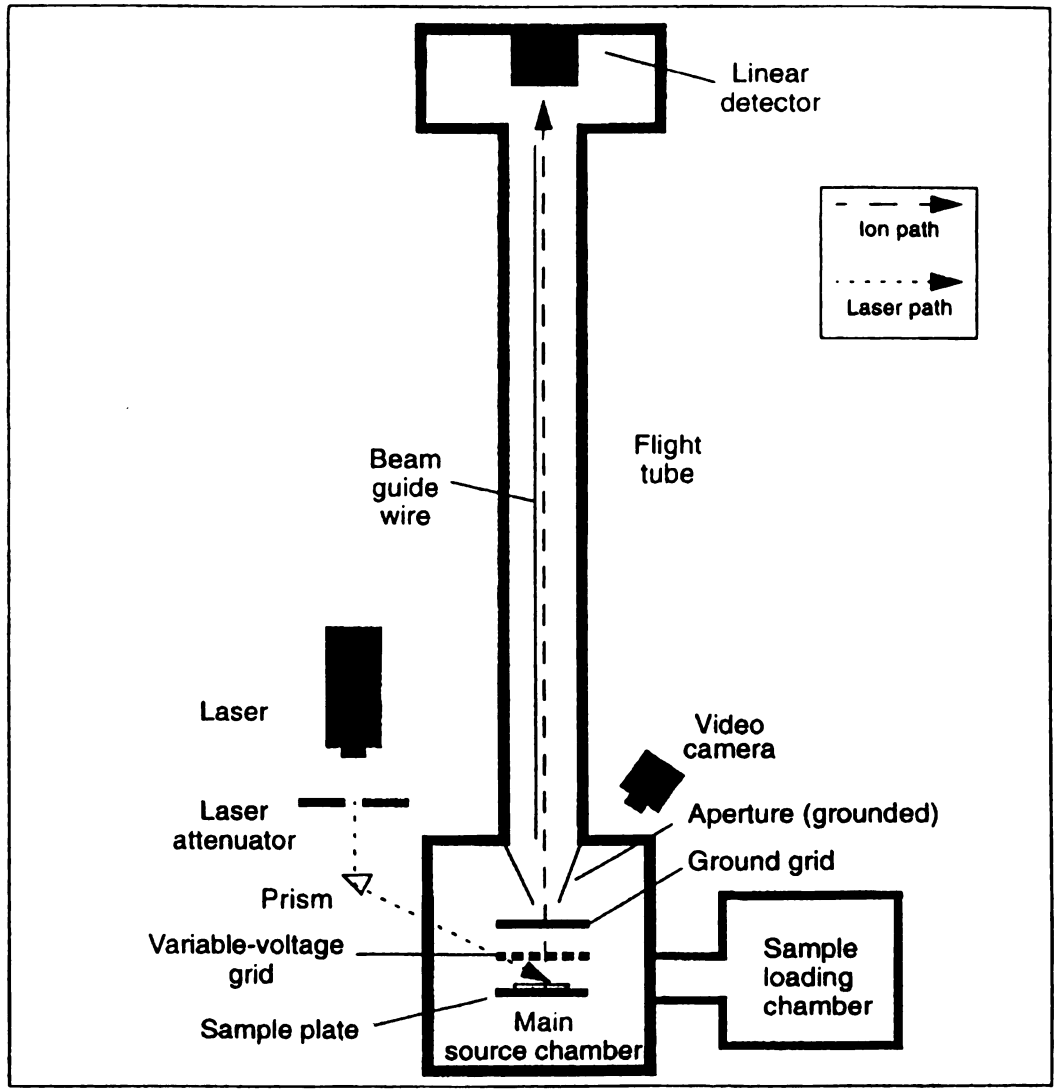


Figure 2.1: Voyager-DE Mass Spectrometer

The analyzer coupled to MALDI is most often time-of-flight (TOF) MS. The separation of different ions with different m/z values is based on their different flight times in the field-free drift region. The basic formula for TOF mass analysis is shown using the following equations:

Kinetic Energy: K.E. = $\frac{1}{2}$ $mv^2 = qEs$

Velocity of the ion: $v = (2qEs/m)^{1/2}$

$t=L_d / v= L_d (m/q)^{1/2}/(2Es)^{1/2}$

q: charge on the ion

E: accelerating electric field

L_d: the length of the flight region

t: flight time

s: the length of the accelerating electric field

A problem associated with the linear TOF-MS is poor resolution. Because of the kinetic energy distribution [30], even ions with the same m/z values will reach the detector at different times. A reflectron, shown in Figure 2.2, was designed to solve this problem. After ions travel through the field free drift region, they are decelerated and reflected by an electrostatic field. The slower ions will spend less time in this region and catch up with the faster ions. This can compensate for the initial velocity differences. The reflectron mode can also be used for post-source decay (PSD) studies to get more structural information, by detecting fragment ions.

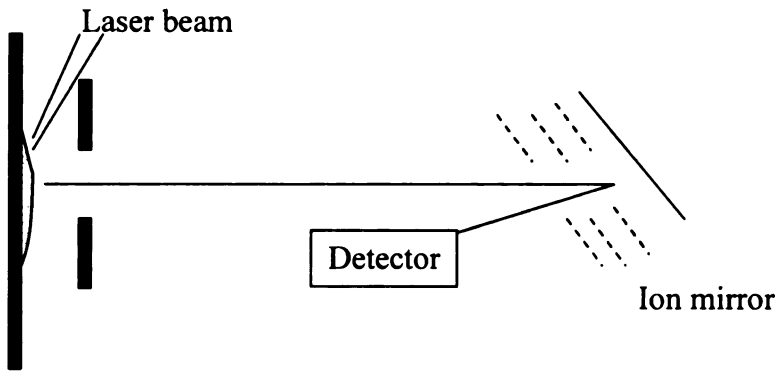


Figure 2.2: Reflectron TOF mass spectrometer

MALDI Experiment

A routinely used MALDI sample preparation procedure is called the "dried-droplet" method. It is performed as follows: take 1 μL of matrix solution, which is usually dissolved in ACN/water (v:v=1:1), and 1 μL analyte solution, which is often dissolved in water. These two solutions are either mixed sequentially on the sample well, or they are mixed first in a vial, then the mixture is applied to the sample plate. The droplet is air-dried and ideally, analyte-doped matrix crystals are formed.

Sample preparation is the most important step for the MALDI experiment. Many research studies have been carried out to investigate the influence of different sample preparation methods on the spectrum [6, 8, 13-18]. The variations such as: matrix, solvent system, pH, and evaporation time are very important for a successful MALDI experiment. A low pH and organic solvents are commonly used in conventional MALDI experiments. They don't correspond to conditions that would preserve the native conformation of proteins. They are the main limitations to the application of MALDI-

MS to non-covalent complex studies. It would be helpful to get a deeper insight into these MALDI parameters when better conditions are explored to study non-covalent complexes.

Matrix

The use of a matrix is a breakthrough to apply mass spectrometry to intact biomolecule analysis. It is believed to serve several functions [28].

1. It isolates analyte molecules. The molar ratio of matrix-to-analyte is usually from 100:1 to 50,000:1 [12]. The presence of a large excess of matrix separates the analyte molecules and thus the strong molecular forces between analytes are reduced.

2. It absorbs energy from the laser and transfers it to the solid crystal lattice. There are mainly two types of laser sources used, UV lasers and IR lasers. Photons in the UV-wavelength range can excite molecules electronically, whereas the irradiation in the IR region will excite them vibrationally or rotationally. Usually, the analyte biomolecules have no absorption at the wavelength of the laser. When the laser energy is deposited in the matrix, it causes the excitation of many matrix molecules. This energy can flow into the crystal lattice, which is usually formed by hydrogen bonds, and heats up a small volume. Both matrix and analyte embedded in the matrix experience a phase transition into the gas phase in a very short time. Figure 2.3 shows the desorption/ionization process. Since the laser is

not directly absorbed by the analyte, they are kept intact. That is why MALDI is characterized by little fragmentation and is called a "soft" ionization technique.

3. It aids in ionization of analyte molecules via a series of photochemical reactions. This will be discussed in the pH section.

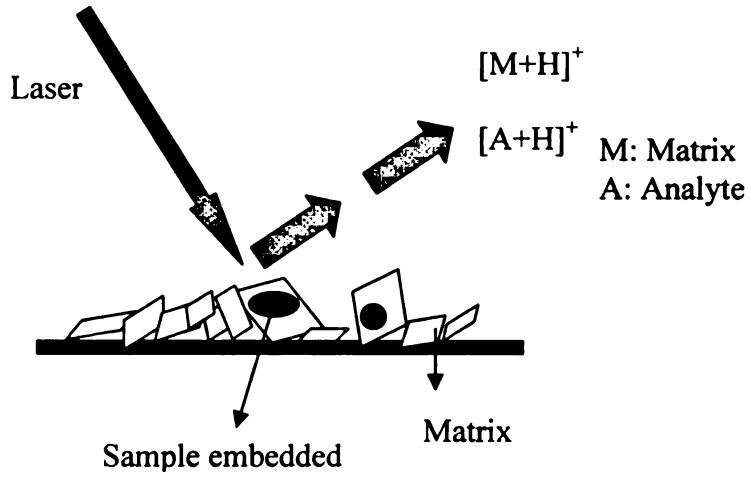
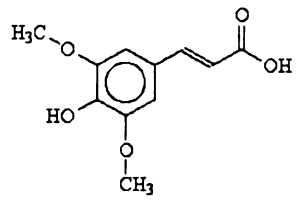


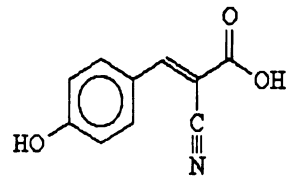
Figure 2.3: Photon absorption by the matrix in MALDI, causing desorption and ionization.

The search for a new matrix candidate is still a trial-and-error procedure. So is the choice of a matrix for a specific analyte. So far, hundreds of matrices have been evaluated. Figure 2.4 shows the structures of several most commonly used matrices. Most of them contain a carboxylic acid group because it may be easy for them to give up a proton during the ionization process. The acidic matrix could be a problem for noncovalent complex study since most proteins tend to denature at low pH. Is the acidic functional group required for a good matrix? Actually, the real importance of the acidic function groups is not established yet. It seems that they are not required for a successful MALDI

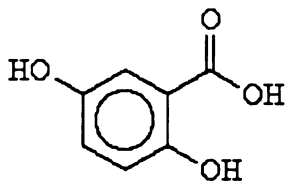
analysis. Matrices without carboxylic acid groups have also been reported [25]. More and more basic matrices have been investigated to meet different goals such as the study of non-covalent complexes [4], because they allow the sample to be prepared under non-denaturing conditions.



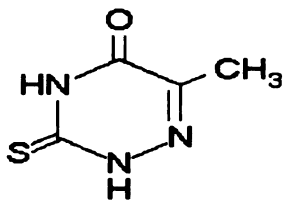
sinapinic acid (SA)



α-cyano-4-hydroxycinnamic acid (CHCA)



2, 5-dihydroxybenzoic acid (DHB)



6-aza-2-thiothymine (ATT)

Figure 2.4: Structures of some most commonly used matrices

Different matrices have very different properties, which have a great influence on the crystallization. For a certain analyte, some matrices yield strong signals but others don't work at all. It is not well understood how matrices behave and how to select a matrix. Based on experience, some general guidelines are known. For example, sinapinic acid is suitable for high molecular weight proteins. However, for a specific analyte, this information is far from sufficient.

The most remarkable property for matrix molecules is their ability to incorporate analyte into their crystal lattice and form analyte-doped matrix crystals. The doping level depends on the affinity of the analyte to absorb to the matrix and the relative diffusion rates of the matrix and analyte [5]. Different matrices have different doping levels for a specific analyte. Solvent composition and crystallization conditions can also alter the doping level. It was found that the doping could be blocked by the presence of contaminants such as salts and detergents [5]. Strupat and coworkers found that the intimate interactions between matrix and analyte are essential for analysis of large analytes in the MALDI process [1].

Different morphology of matrix crystals also influences the MALDI experiment. Under microscopic inspection, many matrices accumulate at the rim of the sample well after crystallization. Only sinapinic acid seemed to evenly distribute around the whole well [19]. Some matrices such as nicotinic acid or sinapinic acid form small crystallites, which are about 10 μm in size. Others such as DHB form needle-like crystals which are several hundred micrometers in length [19]. Sample morphology can differ dramatically depending on what kind of matrix is used. Sample surfaces are heterogeneous, containing two phases: the crystalline matrix phase and the isotropic phase with analyte doped in it [20]. Usually the best result is obtained from the homogeneous area. The heterogeneity can be a potential problem in MALDI.

Matrix has a great influence on fragmentation. Some matrices are classified as "hot" since they are more capable to induce fragmentation. Karas and coworkers found that,

for protonated glycoproteins, post-source decay decreases in the order of SA>DHB>HPA [21]. The energy released from the proton transfer reaction can induce fragmentation. The difference in proton affinity between analyte and matrix can determine the energy available [22]. Proteins and peptides have a higher proton affinity than most of the matrices and thus can extract a proton from a matrix molecule. To detect the intact molecule and prevent fragmentation, a matrix with a higher proton affinity should be chosen. Whether or not the sublimation temperature of matrices also influences the internal energy of the analyte is still under debate [23].

Solvent

The most commonly used solvent in the MALDI experiment is the combination of water and an organic solvent such as acetonitrile, methanol, or ethanol.

There are mainly two reasons to use organic solvents:

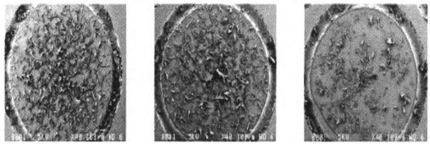
1) Most matrices are organic molecules and are not very soluble in water. The addition of organic solvents can increase the solubility of the matrix [6].

2) The use of organic solvents can facilitate rapid crystal growth. Some organic solvents are volatile and evaporate very quickly. This can speed the crystallization process [7] and have a significant influence on the analyte distribution in the MALDI crystals [26].

The crystal growth in MALDI experiment is quite different from that under equilibrium conditions. Carroll and Beavis [5] addressed the relationship between the speed of crystal growth and doping levels. In the case of mother liquor where crystals grow very

slowly, the rate of protein diffusion is greater than the rate that protein is removed from the solution into the growing matrix crystal. But the protein molecule has to be able to stay long enough on the crystal surface to get trapped inside it. The doping level in this case will be limited by the rate of adsorption of proteins to the growing crystal. Another extreme case is, the crystals grow very fast with respect to protein diffusion, and the doping level will only depend on the bulk concentration of the protein present. In the MALDI experiment, we usually take the latter one, so the proteins included in the matrix crystals is at approximately the same molar ratio of matrix to analyte as in the original solution. Conditions such as the composition of water and organic solvents, and the molar ratio of matrix to analyte, can be manipulated to alter the doping level and thus obtain the best result.

A study of the effect of the different composition of water and ACN on the crystals and mass spectra was done by Vaidyanathan *et al.* [8]. Different combinations of water and ACN were tested. Figure 2.5 shows the distribution of sinapinic acid matrix crystals on the MALDI-MS target wells as observed by scanning electron microscopy. Solvent a) contains more organic solvent and evaporates very fast. It appears to induce smaller and finer crystals. Solvent c) contains more water and evaporates more slowly. The resulting crystals are larger and feather-like. Those grown in solvent b) contain both crystal types but the smaller ones predominate. These experimental results show that smaller crystals yield better spectra than the bigger ones.



a) b) c)

Figure 2.5: Distribution of sinapinic acid matrix crystals on the MALDI-MS target wells as observed by scanning electron microscopy

Despite these advantages of fast crystallization, slow crystallization techniques are used in some case to overcome suppression effects on the matrix crystallization process causing by involatile additives [27].

Water must be present to maintain surface tension. Small organic molecules have very small surface tension and the sample will spread. This will cause a dilution effect and induce irregular crystal formation.

pH

In most MALDI experiments, trifluoroacetic acid or formic acid is used. The use of acid can help the dissolution of some analytes [9]. Trifluoroacetic acid is also found to improve the sample homogeneity in some instances.

The second reason that acid is used is to maintain a low pH environment. Most matrices discovered so far are carboxylic acids and they become ions when the pH is higher than

their pK_a values. Their salts crystallize quite differently from the free acid form of the molecule [10, 11].

Another reason is concerned about the MALDI mechanism. The MALDI mechanism is still under debate. One approach made by Ehring *et al.* is the photochemical ionization model [24]. This model assumed that the ion-generation in UV-laser desorption/ionization (LDI) is initiated by photoionization and formation of radical ions that then react with neutrals to form the final ions. Extending this approach to MALDI, they proposed that the matrix has to form gas-phase acidic ions. They act as proton donors and transfer a proton to analyte molecules [21].

$$MH^+ + A \rightarrow M + AH^+$$

To fully protonate the matrix, a low pH is preferred.

But that is not the only way for the matrix to get protonated. Excited-state proton transfer (ESPT) is also a popular model. The exited matrix molecule is more acidic than when in its ground state. It is easier to give up a proton to the nearby analyte or matrix.

$$M + h\gamma \rightarrow M^*$$

$$M^* + A \rightarrow (M\text{-}H)^- + AH^+$$

$$M + M^* \rightarrow (M\text{-}H)^- + MH^+$$

MALDI is such a complex phenomenon that no unified model has yet been set up to explain it. It comprises the formation of protonated, cationized and even radical species.

Karas *et al.* suggested that this process is relatively independent of the matrix, solvent composition, solution pH, and analyte acid-base properties [10].

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Chapter Three: Review of Study of Cytochrome c Oxidase Using Mass Spectrometry

Introduction

Cytochrome c oxidase (CcO) is the terminal enzyme in the respiratory chain in both mitochondria and bacteria. Cytochrome c oxidase contains 4 redox active metal centers to catalyze the process. The electrons from cytochrome c are accepted by Cu_a, and then transferred to heme a, finally to heme a_3 and the Cu_b binuclear center, where the molecular oxygen gets reduced and water is produced. The biological process is summarized in Figure 3.1.

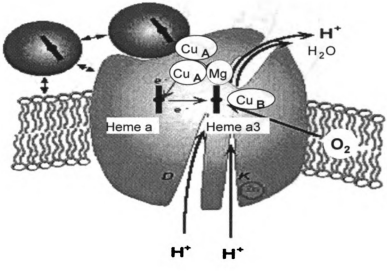

Figure 3.1: Biological function of cytochrome c oxidase
(Picture adapted from: http://www.bch.msu.edu/research/ppg/cyox.htm)

The polypeptide composition of CcO is variable, depending on the evolutionary stage of the organism. 2-4 subunits have been identified in bacteria, 9 in yeast and 13 in mammalian tissues [11]. Figure 3.2 shows the ribbon structure of the 13-subunit enzyme. Subunits I, II, and III are the three largest subunits. Surprisingly, they are similar in all the members of this heme-copper oxidase superfamily. They are believed to be the most important functional subunits in CcO. Organism at higher evolutionary stages contains a number of small subunits, which are thought to help stabilize and modulate the activity of the enzyme [5].

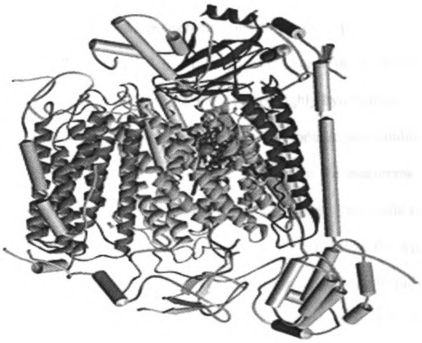

Figure 3.2: 13-Subunit cytochrome c oxidase structure
(Picture adapted from: http://www.msu.edu/~hillier/MF_CcO%20pics.htm)

Over the last years, many analytical techniques such as EPR, and absorbance and resonance Raman spectroscopies, have been used to improve the understanding of the structure and function of this enzyme [7]. With the development of soft ionization techniques yielding little or no fragmentation, such as matrix-assisted laser desorption/ionization and electrospray ionization, mass spectrometry is becoming more and more important in the analysis of large biomolecules including CcO. The study of CcO has been mostly reported by MALDI, since MALDI has a higher tolerance of buffers and salts than ESI.

The Study of Cytochrome c Oxidase Using Mass Spectrometry

CcO presents a challenge to mass spectrometry due to its hydrophobicity. CcO is a membrane protein, usually embedded in lipid bilayers. Some of its domains are in contact with the alkyl chains of the lipids, since they are highly hydrophobic. The other regions that are exposed to the aqueous phase are more hydrophilic and soluble in water. Subunits I, II, and III from bovine heart are mainly located in the membrane space. If CcO falls apart in solution, hydrophobic subunits may aggregate. This could make them difficult to detect by MS. Detergents can be used to help solubilize the hydrophobic proteins. It was found that the detergent lauryl maltoside is a good one to fully disperse CcO [9]. Fortunately, it is a nonionic detergent and is compatible with MALDI analysis. Table 3.1 shows the relative effects of detergents on signal quality in MALDI [15]. The study proved that ionic detergents are detrimental to MALDI MS analysis. They might interfere with the crystallization process. Nonionic detergents can be tolerated at relatively higher concentrations without degrading the spectrum.

Table 3.1: The Relative Effects of Detergents on Signal Quality in MALDI MS [15]

Class	Effect on MALDI spectrum
1	no bad effect: may improve results in mixtures
2	little effect
3	spectrum quality & signal intensity reduced
4	spectrum supressed: detergent must be removed

Detergent	**Class**
n-octyl-glucoside	1
n-dodecyl-glucoside	1
octanoyl-N-methylglucamide	1
decanoyl-n-methylglucamide	1
n-dodecyl-beta-D-maltoside	2
octylphenolpoly(ethyleneglycolether)$_{10}$ (Triton X-100)	3
octylphenolpoly(ethyleneglycolether)$_7$ (Triton X-114)	3
polyethylene glycol (PEG 2000)	3
dodecylpoly(ethyleneglycolether)$_9$ (Thesit)	4
isotridecylpoly(ethyleneglycolether)$_8$	4
CHAPS	4
CHAPSO	4
n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate	4
sodium dodecylsulfate (SDS)	4

The investigation of CcO using mass spectrometry has become even more complicated since the purification of the oxidase by different methods (even from the same tissue) yields enzymes that may differ in subunit composition, aggregating state, and phospholipids content [5]. The different starting forms make it very hard to find a consistent MALDI condition that can be applied to the detection of the enzyme that has been purified using different methods. Studies done so far have proven the success of different matrices for MS detection of the enzyme, purified in different ways.

Cytochrome c oxidase purified from *R. sphaeroides* was studied by Ghaim *et al.* [1]. Three subunits were detected. The enzyme sample was in 0.05% lauryl maltoside, 100 mM potassium phosphate buffer and pH 8.0. The original concentration of the enzyme was 50 μM. In the MALDI experiment, the enzyme was diluted 50:1 with water. Matrix was prepared by dissolving 1.5 mg of HABA([2-(4-hydroxyphenylazo (benzoic acid)] in 1 mL of solvent containing 66% acetonitrile, 33% water, 0.1% TFA. The calculated molecular weights for the 3 subunits are: subunit I: 63,986; subunit II: 32,940; subunit III: 30,139. Singly-charged subunits I and III were clearly detected, as were their doubly and triply charged species. Subunit II can be possibly assigned to a peak at m/z 29,947, although it is less than the expected value by 2993 Da, possibly due to loss of 25 amino acid residues [1]. Their spectra are shown in Figure 3.3.

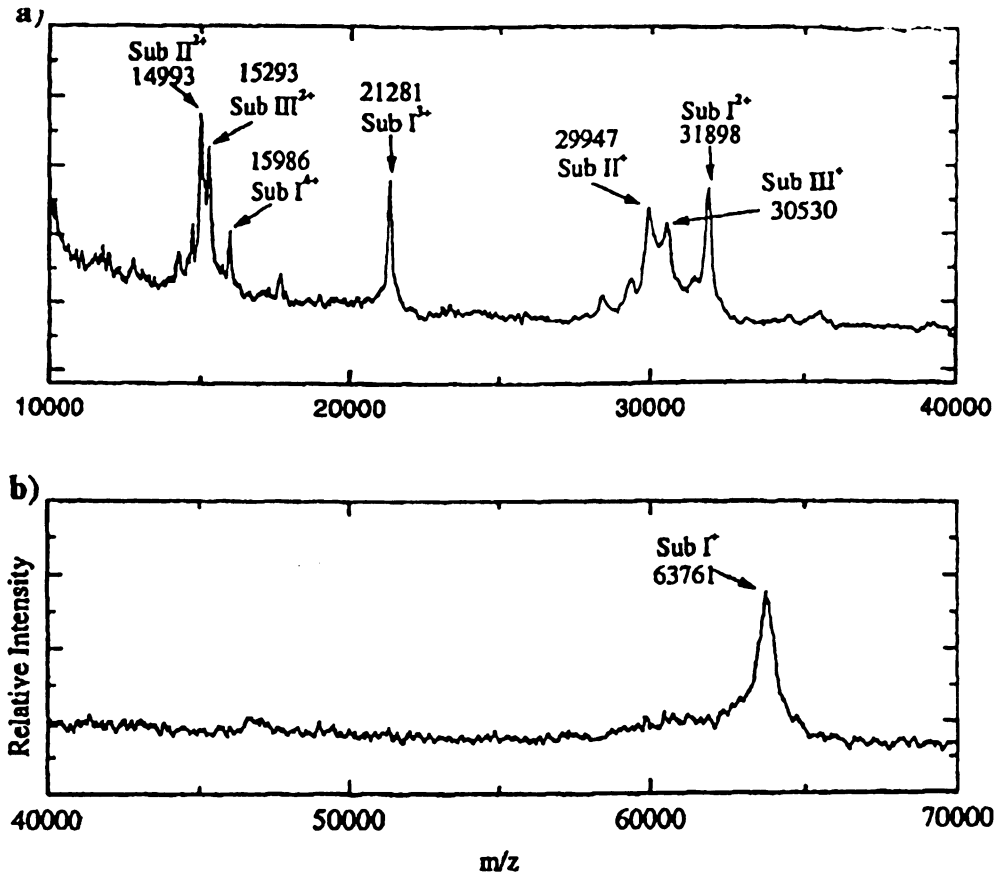

Figure 3.3: MALDI mass spectrum of the Ni^{2+}- NTA resin purified His-tagged aa_3-type cytochrome c oxidase complex from R. *sphaeroides*. a) MALDI spectrum of subunits with less than 40,000 Da molecular weight. b) MALDI spectrum of subunits larger than 40,000 Da molecular weight.

Our lab also studied CcO from *R. Sphaeroides* in which four subunits were observed in MALDI. The starting enzyme solution contained 10 mM KH_2PO_4, 1 mM EDTA, 0.2% lauryl maltoside, pH 7.2. The matrices used in our experiments were made using 1:1 acetonitrile/water solution. When sinapinic acid (SA) was used as matrix, only subunits I, II, and III were detected in the MALDI mass spectrum. Subunit IV was absent. When 2,5-dihydroxybenzoic acid(DHB) was used instead, subunit IV was detected, both the processed form and unprocessed form. But each of these two versions is 131 Da lower

than the expected value, which is due to the loss of the N-terminal methionine. The spectrum from ESI-FTMS of subunit IV is in good agreement with the result from MALDI-MS.

The mammalian enzyme, containing 13 nonidentical subunits, has been studied by high resolution SDS-polyacrylamide gel electrophoresis. It is by far the most complex membrane protein to have its structure solved [13]. The three largest subunits are encode in the mitochondrial DNA and are the core of the enzyme. The smaller ten subunits are nuclear-coded [6].

The earliest study of CcO subunit components from bovine heart in mass spectrometry was carried out by Schindler *et al.* [8]. Subunits VIIIa and VIIIb were isolated from bovine heart and successfully detected in ESI-MS.

CcO isolated from bovine heart and liver was studied by Marx and coworkers [2]. After a series of purification procedures, the final solution contained 0.5 M NaCl, 0.05% lauryl-β-D-maltoside or 1% octyl-β-D-glucopyranoside, 5 mM NH_4HCO_3 buffer, pH 7.6. The matrix used was sinapinic acid. It was prepared as a saturated solution in 3:1 water/acetonitrile.

The results are shown in Table 3.2.

Table 3.2: Molecular Masses of the Subunits of Bovine Heart and Liver Cytochrome c Oxidase

Subunits	Expected molecular weight (bovine heart)	Detected $(M+H)^+$	Expected molecular weight (bovine liver)	Detected $(M+H)^+$
I	56,993	ND	56,993	ND
II	29,918	ND	29,918	ND
III	26,023	ND	26,023	ND
IV	17,153	ND	17,153	17,156
V (Va)	12,436	12,436	12,436	12,436
VI (Vb)	10,670	10,670	10,670	10,670
VII (VIb)	10,063	10,066	10,063	10,066
VIII (VIa)	9,436	ND	9,539	9,541
IX (VIc)	8,479	8,524	8,479	8,520
X (VIIb)	6,674	6,676	6,619	6,620
XI (VIIa)	6,357	6,358	6,357	6,357
XII (VIIc)	5,441	5,442	5,441	5,440
XIII (VIII)	4,962(Lys) 4,992(Arg)	4,962 4,992	5,048	5,047

Most of the subunits were detected except subunits I, II, III, IV, and VIII for the enzyme from bovine heart and subunits I, II, and III for the enzyme from bovine liver. The reason they suggested for the disappearance of subunits I, II, and III was, those hydrophobic subunits tend to aggregate even in the presence of detergent and are no longer accessible for MALDI-MS. Most of the molecular weights of the detected subunits match the published values very well except that some corrections were made for subunits IX and X.

Improved results were obtained by Musatov [3]. The protein (25 μM) was solubilized in 20 mM tris-SO_4 buffer, pH 7.4 containing 2 mM dodecyl maltoside. The molecular weight of each subunit was verified by MALDI-MS and ESI-MS. For the ESI-MS analysis, samples that elute from HPLC were mixed with 50% acetonitrile/0.5% acetic acid to yield 2 μM protein. 11 subunits eluted from the reversed-phase HPLC and were detected by ESI-MS. However, the spectrum was not shown. The two most hydrophobic subunits, I and III, precipitate on the column and thus are not detected. For the MALDI experiment, 1μL of a solution containing 2 μM CcO and 20 mM dodecyl maltoside was mixed with SA dissolved in acetonitrile/TFA (v:v=1:1) on the sample plate. All of the 13 subunits were observed in MALDI. Compared with Marx's result [2], the improved MALDI spectrum might be due to the increased concentration of lauryl maltoside.

The solution conditions such as the type of detergent used and its concentration, the buffer and other components in the solvent, are all essential for the study of cytochrome c oxidase in mass spectrometry. That could explain why different matrices are most useful for analysis of the enzyme from the same tissue, but in solutions resulting from different purification procedures.

Although the polypeptide composition is the main part of this enzyme, the other components can not be ignored. The heme group, metals, and phospholipids are all tightly associated with the protein and thus should also be considered as part of the enzyme. Six phospholipids have been resolved in the crystal structure from *R. Sphaeroides* CcO [4]. The amount of lipids found associated with the oxidase complex depends on the purification methods. In our lab, Distler *et al.* [4] did a broad mass spectrometric analysis of lipid and heme components of CcO from *R. Sphaeroides.* In the study, the improved detection of lipid was achieved by using DHB as matrix dissolved in1:1 acetonitrile/water. The masses of the lipids and the type of lipids present were determined in reflectron mode MALDI-MS. The structural information was obtained by running post-source decay (PSD) analysis. MALDI-MS was demonstrated to be a strong tool to monitor the lipids content after various levels of purification of the enzyme. In addition, a peak at m/z 852 was confirmed to represent the heme group by a PSD experiment.

Another challenge CcO presents to mass spectrometric analysis is, it is a noncovalent complex of several different subunits. For CcO from bovine heart, the molecular weight

for the monomer calculated for the protein moiety is 204,005 Da and that for the other constituents (except for the cholic acid) identified in the electron density map is 6,998 [6]. Although it was expected to see one peak representing the whole enzyme, the complex completely dissociated in the MALDI experiment and only individual subunit was detected. How and where the complex fell apart in the MALDI experiment is still unclear. The interaction with matrix, organic solvent and low pH used in the MALDI experiment could all make contributions. It was found that some organic solvents denature proteins including cytochrome c oxidase [10]. pH also has an effect. The optimal pH range for the enzyme to maintain its activity is 5.0 to 8.5. Evidence showed that protein denaturation occurred at pH 4.5 [12]. Previous studies showed that some compounds such as fucose [14] can be used in the MALDI experiment as an additive to help stabilize the noncovalent complex in the harsh MALDI sample preparation conditions. Distler *et al.* reported the detection of the intact enzyme from *R. Sphaeroides* by the addition of sucrose. The spectrum is shown in Figure 3.4. Several peaks were observed representing the complexes of CcO subunits [4].

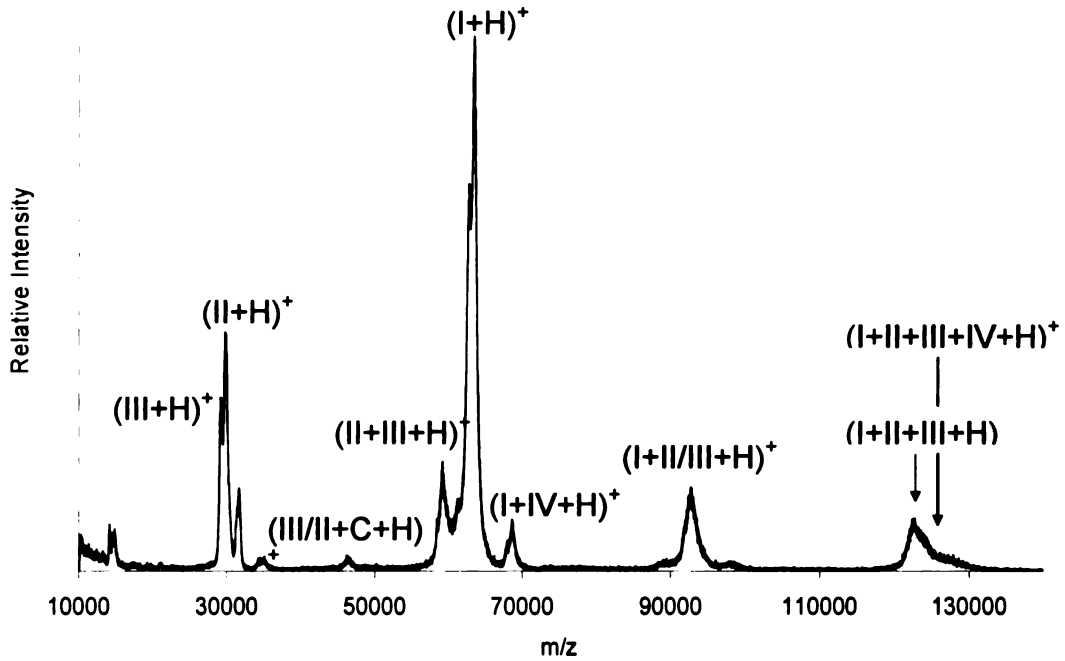

Figure 3.4: Positive-ion linear MALDI-TOF spectrum of cytochrome c oxidase from *R. Sphaeroides*. SA/sucrose was used as the matrix.

Besides the protein subunits and other components, there are also some other studies that have been carried out on CcO. In Marx's study, 7-diethyl-amino-3-(4'-maleimidylphenyl)-4-methylcoumarin was used to investigate cysteine status [2].

Mass spectrometry was also used to investigate the chemical modification of the enzyme [3]. Bovine heart enzyme was incubated in lipid peroxidation product 4-hydroxy-2-nonenal (HNE) and then studied by MALDI-MS and ESI-MS. Both of these methods showed that 6 subunits were detected as HNE additives, subunits II, IV, Vb, VIIa, VIIc

and VIII. Identification of the HNE reaction site in subunit VIII was achieved by ESI tandem ESI-MS analysis.

The study of cytochrome c oxidase using mass spectrometry is far from being mature. The challenge it placed on mass spectrometry will give us a great opportunity to investigate better conditions to apply MALDI-MS and ESI-MS to membrane protein analysis. The use of detergents, the presence of other components in the enzyme and the stabilization of the whole noncovalent complex remains to be further studied.

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Chapter Four: The Detection of Cytochrome C Oxidase Subunits Using Mass Spectrometry

Experimental Section

I worked with several different bovine heart cytochrome c oxidase (CcO) samples, which contained different buffers and detergents.

Sample A was isolated from bovine heart using the Yoshikawa purification procedure with a minor modification (See Appendix A). The enzyme is in a 0.2 M phosphate buffer (pH=7.4), 25% $(NH_4)_2SO_4$ and 0.5% sodium cholate.

Sample B was purified, using an Ultrafree-15 centrifugal filter (Millipore, Bedford MA) from sample A in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)-KOH (pH 7.4), 15 mM KCl and 0.01% lauryl maltoside.

Sample C contains 10 mM phosphate buffer (pH=7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 180 mM KCl and polyethylene glycol dodecyl ether (Brij-35) (concentration is unknown). The concentration of CcO is 108 μM, which was determined by UV-VIS spectrometry.

The compounds, 2, 5-dihydroxybenzoic acid (DHB), 6-aza-2-thiothymine (ATT), sinapinic acid (SA), and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (St. Louis, MO). Standard solutions of these MALDI matrices were prepared using water/acetonitrile (v:v=1:1). Water was used to dilute the CcO samples to 5 μM. Equal volumes of matrix and protein solutions were deposited on the MALDI

steel sample plate. Calibration mixtures 2 and 3, purchased from PerSeptive Biosystems (Framingham, MA), were used for calibration.

Reversed-Phase High Performance Liquid Chromatography: the HPLC analysis of CcO subunits was performed using gradient elution from a C_{18} reversed phase column and a Microm HPLC system. The gradient was made from mixtures of solvent A (5% [0.1% formic acid] and 95% ACN) and solvent B (95% [0.1% formic acid] and 5% ACN).

Linear MALDI mass spectra were recorded on a PerSeptive Biosystems (Framingham, MA) Voyager delayed extraction (DE) time-of-flight (TOF) mass spectrometer with a nitrogen laser (337 nm, 3 ns pulse length). Typically 50 laser shots were averaged for each spectrum. The accelerating voltage was 20 kV, the delayed time was 500 ns, the grid voltage was 94% of the accelerating voltage, and the magnitude of the guide wire voltage was 0.1% of the accelerating voltage.

FTMS spectra were acquired on a Bruker Daltonics (Billerica, MA) Apex III mass spectrometer with a 7 Tesla superconducting shielded magnet.

Results and Discussion

UV Absorption Spectra

The concentrations of cytochrome c oxidase samples were determined by UV spectrometry. Figure 4.1 shows the UV spectra of oxidized and reduced forms of the purified CcO enzyme sample C. The spectrum of the oxidized form (spectrum a) has an

absorption peak at 416 nm and the reduced form obtained with sodium dithionite has absorption peaks at 440 nm and 602 nm (spectrum b).

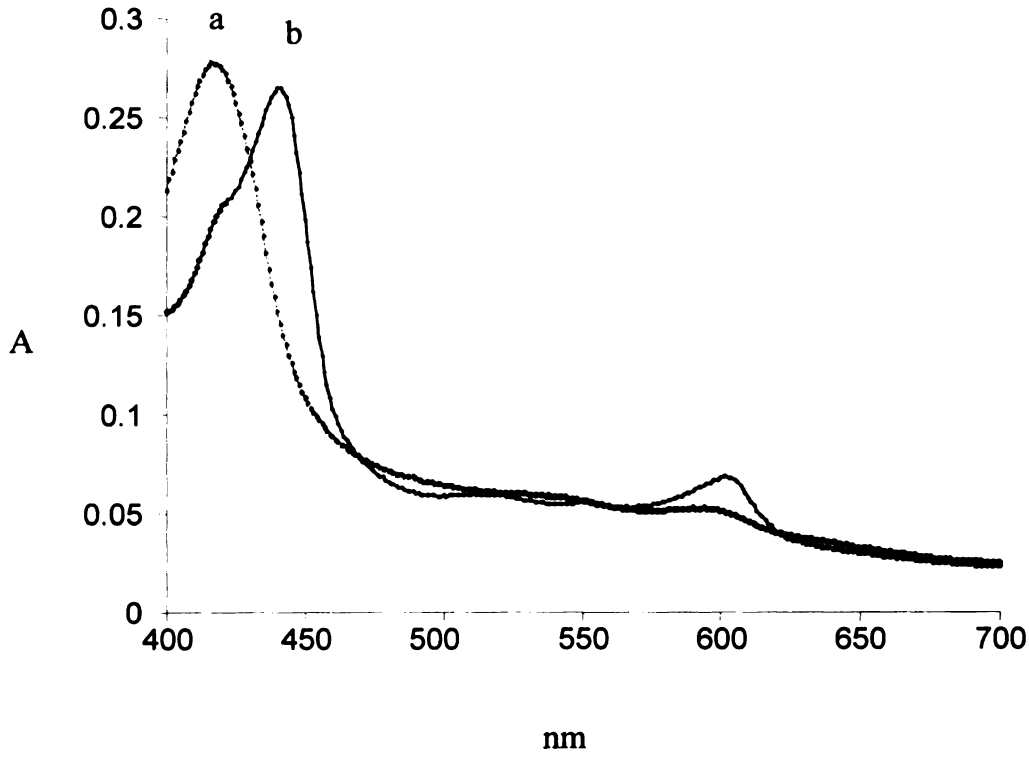


Figure 4.1: a.) Absorption spectrum of oxidized form of cytochrome c oxidase;
b.) Absorption spectrum of reduced form of cytochrome c oxidase (reduced with $Na_2S_2O_4$)

The protein concentration can be calculated using the Lambert-Beer law:

$A = \varepsilon bC$

A: absorption

ε: extinction coefficient (37,000 L $cm^{-1}M^{-1}$ for cytochrome c oxidase))

b: thickness of the cell (0.05 cm in our experiment)

C: concentration

The absorption at 602 nm in spectrum b is 0.07044. The spectrum of the oxidized form (spectrum a) has no absorption at 602 nm and thus can be used as the background, which is 0.05.

$A = \varepsilon bC$

That is: $(0.07044-0.05) = 37{,}000\ L\ cm^{-1}M^{-1} * 0.05\ cm * C$

The concentration of cytochrome c oxidase can be determined as 108 pmol/μL.

The concentration of the other cytochrome c oxidase samples can be determined using the same method.

The Detection of Cytochrome c Oxidase Subunits Using MALDI TOF MS

The three largest subunits of cytochrome c oxidase (CcO) are encoded in the mitochondrial DNA and are the core of the enzyme. The smaller ten subunits (subunits IV to XIII) are nuclear-coded. In the conventional MALDI experiment, all of the ten nuclear coded subunits were detected using MALDI TOF MS. Figure 4.2 shows the spectrum obtained for cytochrome c oxidase sample C using sinapinic acid (SA) as the matrix. Among the tested matrices, SA is the best for the detection of the ten smaller subunits in terms of the resolution and signal intensity. The spectrum is easy to interpret, as the predominant peaks are the protonated molecules of these subunits. No fragmentation was observed. These peaks can be used to determine the molecular weight of each subunit. The results are summarized in Table 4.2. However, the problem with SA is the adduct formation. Small peaks due to SA attached to subunits were observed. For example, the inset of Figure 4.2 shows the peak representing protonated subunit XIII

at m/z 4,964. Beside it, a peak at m/z 5,172 was detected. The mass difference between these two peaks is 208, which corresponds to the molecular weight of sinapinic acid.

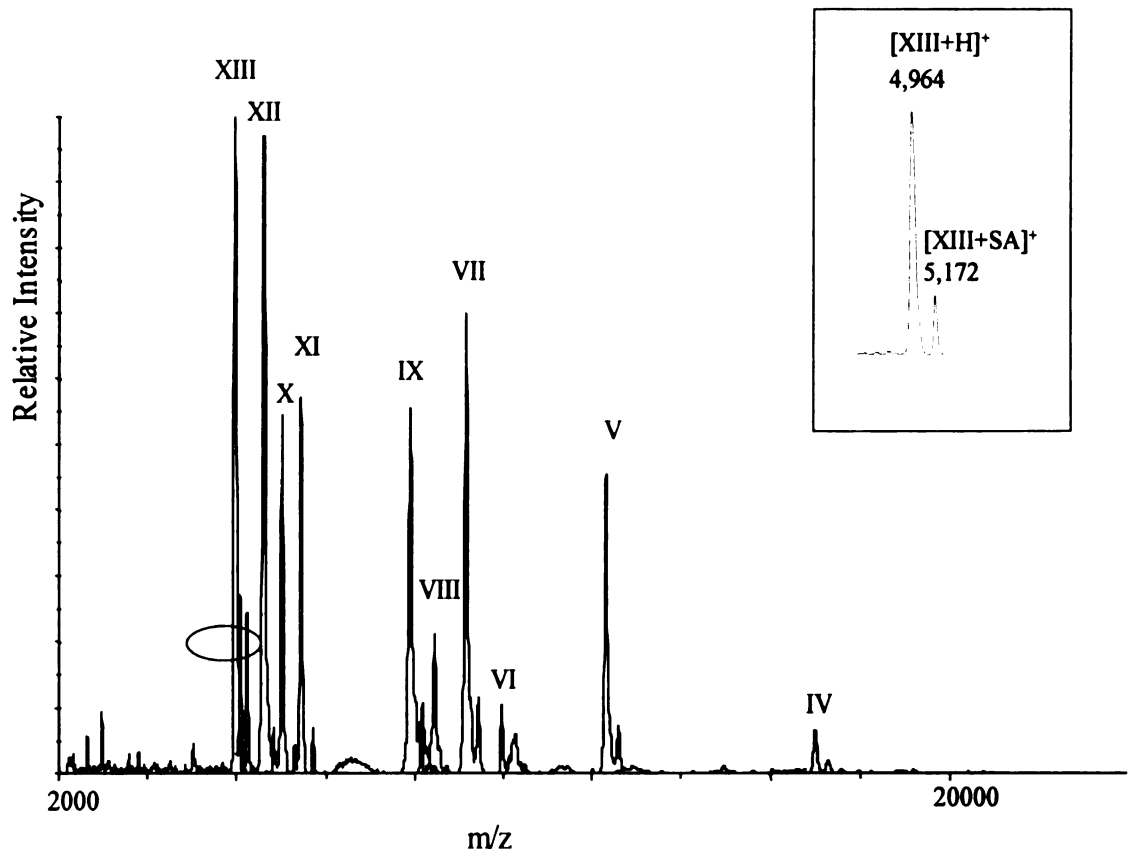


Figure 4.2: Positive-ion linear MALDI-TOF spectrum of cytochrome c oxidase. The ten nuclear coded subunits were detected. SA was used as matrix.

The Detection of Cytochrome c Oxidase Subunits Using ESI-FTMS

Compared with MALDI, ESI has a lower tolerance for impurities such as detergents, buffers and salts. CcO sample C was analyzed using ESI-FTMS and only peaks

representing singly charged detergent molecules were detected. The reason might be due to the presence of a high concentration of detergent. To eliminate the detrimental effects of the detergent, CcO was purified using reversed-phase high performance liquid chromatography. The HPLC chromatogram is shown in Figure 4.3.

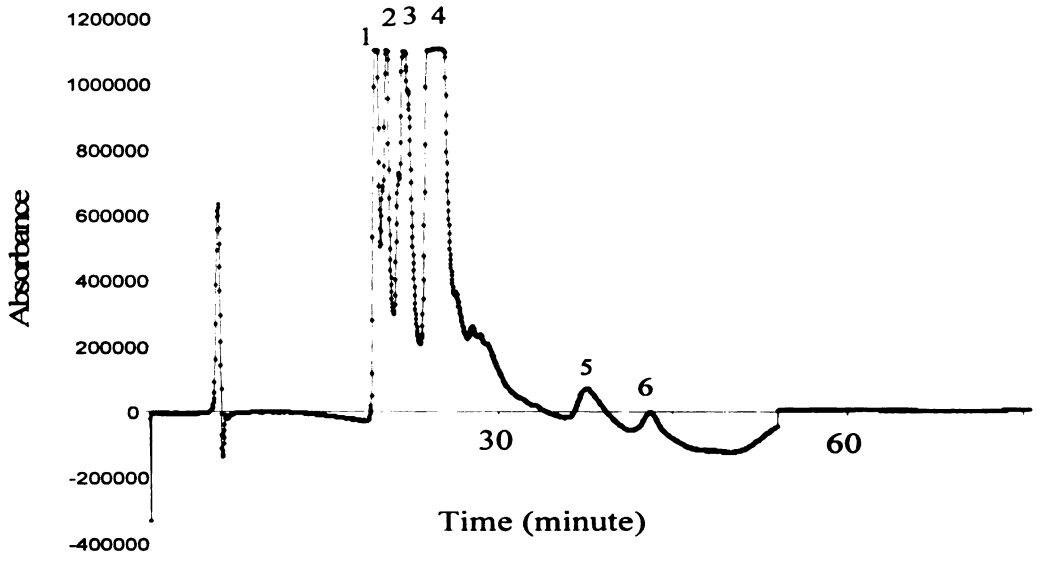


Figure 4.3: HPLC chromatogram of CcO.

The solution that eluted from the HPLC was introduced into the electrospray ion source using a syringe pump at a rate of one micromolar/minute. The spectra obtained by ESI-FTMS were deconvoluted and many peaks were observed. Figure 4.4 shows the deconvoluted spectrum of the fraction eluted from the HPLC at 20 minutes. However, only a few of these peaks have isotopic distributions. For example, if we zoom in on the peak around 4,960, a set of isotopic peaks is observed as shown in Figure 4.4. The

isotope distribution can help us determine which of these deconvoluted peaks actually represent a true species.

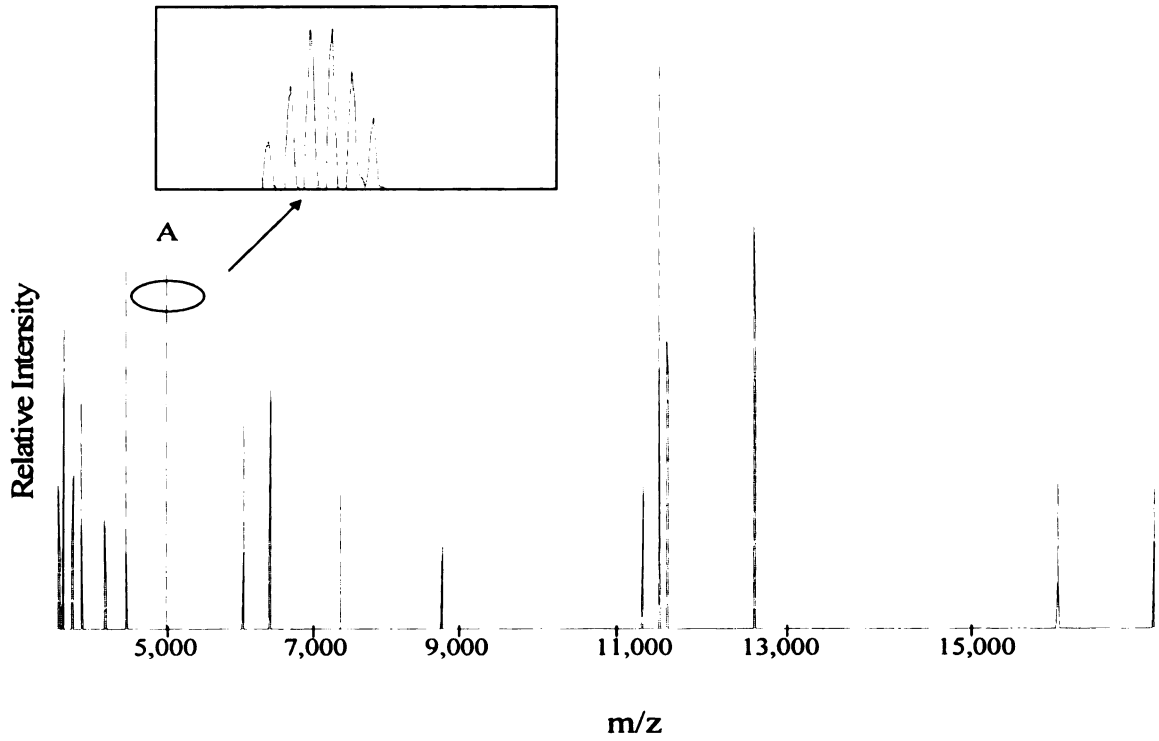


Figure 4.4: Deconvoluted ESI-FTMS spectrum of cytochrome c oxidase eluted from HPLC at 20 minute.

Table 4.1 summarized all the peaks that have a set of isotopes in the deconvoluted ESI-FTMS spectra of fractions collected from HPLC and their masses.

Table 4.1: Isotope Distribution Peak List for All the Peaks Observed in the Deconvoluted ESI-FTMS Spectrum

Peak A (m/z)	Peak B (m/z)	Peak C (m/z)	Peak D (m/z)	Peak E (m/z)
4957.2045	5436.4119	6352.6384	6668.6893	7504.8336
4958.3002	5437.4722	6353.6658	6669.7213	7505.8708
4959.2949	5438.5006	6354.6963	6670.7480	7506.9162
4960.3258	5439.5052	6355.7200	6671.7726	7507.9551
4961.3281	5440.5233	6356.7595	6672.7833	7509.0003
4962.3630	5441.5279	6357.7105	6673.7901	7510.0412
4963.4429	5442.5622	6358.7238		7511.0836
		6359.7960		

Peak F (m/z)	Peak G (m/z)	Peak H (m/z)	Peak I (m/z)	Peak J (m/z)
8514.5227	9942.1964	10041.6835	10055.6054	11352.6916
8515.4653	9943.1704	10042.6751	10056.7167	11353.9618
8516.4898	9944.1430	10043.6686	10057.8310	11355.3582
8517.5481	9945.1156	10044.6627	10058.9472	11356.7659
8518.6178	9946.3154	10045.6572	10060.0595	11358.1727
8519.5989	9947.2975	10046.6521	10060.9132	11359.4670
8520.6478	9948.2701	10047.6480	10062.0241	11360.8719
8521.7085	9949.2441	10048.6412	10063.1392	11362.2854
8522.7783	9950.2214	10049.6340	10064.2565	11363.6982
	9951.4239	10050.6310	10065.3694	
	9952.4021	10051.7079	10066.2225	
		10052.7054	10067.3373	

Peak K (m/z)	Peak L (m/z)	Peak M (m/z)	Peak N (m/z)
11392.4589	12426.6438	12290.7631	13076.0436
11393.5167	12427.6141	12292.2669	13077.4887
11394.3728	12428.6788	12293.4870	13078.6609
11395.3990	12429.7340	12294.7044	13080.1064
11396.3789	12430.7552	12295.9366	13081.2788
11397.4204	12431.7966	12297.1391	13082.7291
11398.2673	12432.8371	12298.3579	
11399.2867	12433.8763	12299.8567	
11400.3017	12434.9200	12301.0776	
	12435.9873	12302.2986	

Some of the peaks listed in Table 4.1 could possibly represent cytochrome c oxidase subunits. For example, the m/z 4960 peaks in the deconvoluted spectrum (Figure 4.5 a)) are very close to the molecular weight of subunit XIII. To determine if this peak really represents subunit XIII, we compared this spectrum with the theoretical isotope distribution of subunit XIII that is shown in Figure 4.5 (b). They are very similar, but the peak with the highest relative intensity detected in the deconvoluted ESI-FTMS spectrum is 1.3 Da off the theoretical value. This is most probably due to a systematic error. Meanwhile two isotope peaks that have the lowest relative intensity are not detected.

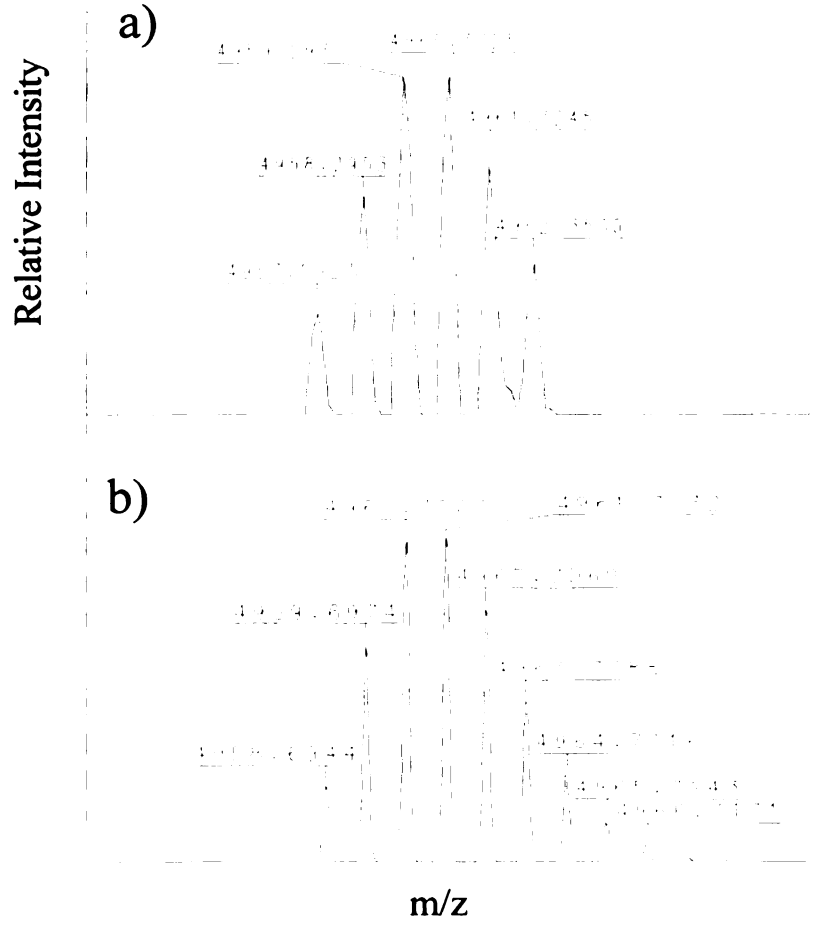


Figure 4.5: a) Deconvoluted ESI-FTICR spectrum. B) Theoretical isotope distribution for subunit XIII

By comparing the deconvoluted spectrum with the theoretical isotope distribution, peak A, B, C, D, F, I, and L in Table 4.1 can be determined to represent CcO subunits XIII, XII, XI, X, IX, VII and V respectively. The molecular weight information determined from ESI FTMS confirmed the results obtained from MALDI-TOF-MS. Other peaks in Table 4.1 are not completely identified yet. Their masses don't match the molecular weight of any subunit. Some of them might represent some subunits with some amino acid residues falling off. For example, the average m/z value of peak G is around 9,948. It is 77 mass units lower than the molecular weight of subunit VII, which is 10,025 calculated from the amino acid sequence. Considering the systematic error, the mass difference is very close to the molecular weight of the residue mass of alanine. The alanine is the C- terminal residue of subunit VII. So peak G could represent subunit VII with alanine residue falling off.

Table 4.2 summarized the results from MALDI MS and ESI FTMS. Most of the molecular weights of the CcO subunits detected in MALDI-TOF-MS and ESI-FTMS match the values calculated from the reported amino acid or DNA sequence very well except for subunits VII, VIII, IX and X. The mass data indicates that the published sequences are correct for subunits IV, V, VI, XI, XII, and XIII. There is no posttranslational modification at these subunits.

Table 4.2: Molecular Weights of Protein Subunits of Bovine Heart Cytochrome C Oxidase

Subunits	Expected molecular weight [a]	MALDI-TOF-MS [f]	ESI-FTMS [g]
I [b]	57, 032	ND [g]	ND
II [b]	29,932	ND	ND
III [b]	26,021	ND	ND
IV [b]	17,152	17, 157	ND
V [b]	12,436	12,436	12,432
VI [c]	10,670	10,671	ND
VII [d]	10,025 [i]	10,063	10,060
VIII [e]	9,436 [i]	9,532	ND
IX [b]	8,479 [i]	8,524	8,518
X [b]	6,244 [i]	6,667	6,671
XI [b]	6,357	6,355	6,355
XII [b]	5,441	5,440	5,440
XIII [b]	4,962(Lys)	4,964	4,961

[a] Molecular masses (Da) were determined either from direct amino acid sequence analysis or by DNA sequence analysis. [b] Reference *14*. [c] Reference *15*. [d] Reference *16*. [e] Reference *17*. [f] Experimentally determined mass (Da) by MALDI-MS. [g] Experimentally determined mass (Da) by ESI-FTMS. [h] ND, not detected. [i] The results from MALDI MS and ESI FTMS aren't in agreement with the values calculated from the reported amino acid or DNA sequence.

In Table 4.2, subunit X shows molecular weights of 6,667 and 6,671 Da in MALDI MS and ESI FTMS respectively, the value calculated from the amino acid sequence reported by Yoshikawa *et al.* [14] being 6,244. The mass difference between the published value

and the value resulting from mass spectrometry is about 423. The error is greater than the molecular weight of an amino acid residue. The molecular weight determined from the gene sequence is 6,674 [19], Which is much closer to our value detected by mass spectrometry (Δ[real mass-expected mass]=7). Yoshikawa's sequences resulting from the crystal structure failed to detect some amino acid residues. The C-terminal residues of their protein sequence of subunit X, -H-G-H-A-S-K-K, must be corrected to –G-W-A-S-F-P-H-K-K.

The same reason can be applied to subunit VIII. The molecular weight detected by MALDI MS is 9,532, the value calculated from the amino acid sequence reported by Yoshikawa *et al.* [14] being 9,436. The difference between these two is 96, which corresponds to the molecular weight of residue phenylalanine (P). This is conformed by the value determined from the gene sequence [17]. Yoshikawa's sequences resulting from the crystal structure failed to detect the C-terminal residue –P, which will result in an additional 97 Da [17].

For subunit IX, The mass difference between the m/z detected by MALDI MS and the expected molecular weight is about 42. The reason might be that the N-terminus could be blocked with an acetyl group, which will give a molecular weight 42 Da higher than the molecular weight calculated from the amino acid sequence. If this is true, the molecular weight of subunit IX will be 8,521, which is very similar to the value we detected by MALDI MS.

For subunit VII, The molecular weight determined by MALDI MS and ESI FTMS 10,063 and 10,060, while the value calculated from the protein sequence is 10,025. The additional 38 mass unit could indicate an acetylation at the N-terminus of this polypeptide chain. But if this is true, the acetylation will result in an addition of 42 mass units. This difference of 4 mass units could be resulted from four cysteine residues forming two disulfide bonds. This will eliminate four hydrogen atoms, which will give a molecular weight four mass units less than the theoretical value. This conclusion is in agreement with the study of Marx *et al.*. It was found that four cysteine residues in subunit VII are involved in the formation of disulfide bridges by the use of 7-diethyl-amino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) [21].

Based on the above discussion, the correct sequences and molecular weights (without methionines oxidized) of the subunits of CcO from bovine heart have been shown in Table 4.3.

Table 4.3: Sequences and Molecular Weights of Protein Subunits of Cytochrome c Oxidase from Bovine Heart

Subunits	Sequence	Molecular Weight
I [a]	MFINRWLFST NHKDIGTLYL LFGAWAGMVG TALSLLIRAE LGQPGTLLGD DQIYNVVVTA HAFVMIFFMV MPIMIGGFGN WLVPLMIGAP DMAFPRMNNM SFWLLPPSFL LLLASSMVEA GAGTGWTVYP PLAGNLAHAG ASVDLTIFSL HLAGVSSILG AINFITTIIN MKPPAMSQYQ TPLFVWSVMI TAVLLLLSLP VLAAGITMLL TDRNLNTTFF DPAGGGDPIL YQHLFWFFGH PEVYILILPG FGMISHIVTY YSGKKEPFGY MGMVWAMMSI GFLGFIVWAH HMFTVGMDVD TRAYFTSATM IIAIPTGVKV FSWLATLHGG NIKWSPAMMW ALGFIFLFTV GGLTGIVLAN SSLDIVLHDT YYVVAHFHYV LSMGAVFAIM GGFVHWFPLF SGYTLNDTWA KIHFAIMFVG VNMTFFPQHF LGLSGMPRRY SDYPDAYTMW NTISSMGSFI SLTAVMLMVF IIWEAFASKR EVLTVDLTTT NLEWLNGCPP PYHTFEEPTY VNLK	57,032
II [a]	MAYPMQLGFQ DATSPIMEEL LHFHDHTLMI VFLISSLVLY IISLMLTTKL THTSTMDAQE VETIWTILPA IILILIALPS LRILYMMDEI NNPSLTVKTM GHQWYWSYEY TDYEDLSFDS YMIPTSELKP GELRLLEVDN RVVLPMEMTI RMLVSSEDVL HSWAVPSLGL KTDAIPGRLN QTTLMSSRPG LYYGQCSEIC GSNHSFMPIV LELVPLKYFE KWSASML	26,021
III [a]	MTHQTHAYHM VNPSPWPLTG ALSALLMTSG LTMWFHFNSM TLLMIGLTTN MLTMYQWWRD VIRESTFQGH HTPAVQKGLR YGMILFIISE VLFFTGFFWA FYHSSLAPTP ELGGCWPPTG IHPLNPLEVP LLNTSVLLAS GVSITWAHHS LMEGDRKHML QALFITITLG VYFTLLQASE YYEAPFTISD GVYGSTFFVA TGFHGLHVII GSTFLIVCFF RQLKFHFTSN HHFGFEAAAW YWHFVDVVWL FLYVSIYWWG S	29,932
IV [a]	AHGSVVKSED YALPSYVDRR DYPLPDVAHV KNLSASQKAL KEKEKASWSS LSIDEKVELY RLKFKESFAE MNRSTNEWKT VVGAAMFFIG FTALLLIWEK HYVYGPIPHT FEEEWVAKQT KRMLDMKVAP IQGFSAKWDY DKNEWKK	17,152
V [a]	SHGSHETDEE FDARWVTYFN KPDIDAWELR KGMNTLVGYD LVPEPKIIDA ALRACRRLND FASAVRILEV VKDKAGPHKE IYPYVIQELR PTLNELGIST PEELGLDKV	12,436
VI [b]	ASGGGVPTDE EQATGLEREV MLAARKGQDP YNILAPKATS GTKEDPNLVP SITNKRIVGC ICEEDNSTVI WFWLHKGEAQ RCPSCGTHYK LVPHQLAH	10,670
VII [c]	AEDIQAKIKN YQTAPFDSRF PNQNQTRNCW QNYLDFHRCE KAMTAKGGDV SVCEWYRRVY KSLCPISWVS TWDDRRAEGT FPGKI (N-terminal is blocked with an acetyl group)	10,067
VIII [d]	ASAAKGDHGG TGARTWRFLT FGLALPSVAL CTLNSWLHSG HRERPAFIPY HHLRIRTKPF SWGDGNHTFF HNPRVNPLPT GYEKP	9,533

IX [c]	STALAKPQMR GLLARRLRFH IVGAFMVSLG FATFYKFAVA EKRKKAYADF YRNYDSMKDF EEMRKAGIFQ SAK (N-terminal is blocked with an acetyl group)	8,521
X [a]	IHQKRAPDFH DKYGNAVLAS GATFCVAVWV YMATQIGIEW NPSPVGRVTP KEWREQ	6358
XI [f]	FENRVAEKQK LFQEDNGLPV HLKGGATDNI LYRVTMTLCL GGTLYSLYCL GWASFPHKK	6,674
XII [a]	SHYEEGPGKN IPFSVENKWR LLAMMTLFFG SGFAAPFFIV RHQLLKK	5,442
XIII [a]	ITAKPAKTPT SPKEQAIGLS VTFLSFLLPA GWVLYHLDNY KKSSAA	4,962

[a] Reference *14*. [b] Reference *15*. [c] Reference *16*. [d] Reference *17*. [e] Reference *18*. [f] Reference *19*.

Why Weren't Subunits I, II and III Detected?

While the conventional MALDI experiment allowed the detection of the ten smaller subunits, the intact CcO complex and the three largest subunits were not detected. Instrument settings were adjusted to achieve better results for the higher mass range, but this alone did not result in the detection of additional peaks. This result was disappointing since subunits I, II and III are the core subunits of cytochrome c oxidase. They are believed to be the most important functional subunits to catalyze the biological process. The question is, why weren't the three core subunits of CcO detected?

A possible mechanism is proposed as shown in Figure 4.6. Under the traditional MALDI conditions, the CcO complex dissociates. Cytochrome c oxidase is a membrane protein and contains hydrophobic segments. Subunits I and III are located in the membrane space and are more hydrophobic than the other smaller ones. To minimize the contact

with water and thus increase the enthalpy, these hydrophobic subunits tend to aggregate in solution. Their aggregation makes them no longer accessible to the MALDI analysis.

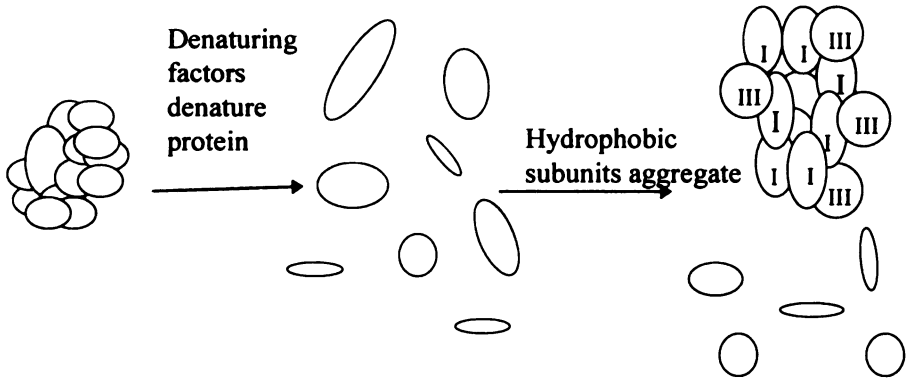


Figure 4.6: Mechanism for the CcO analysis in the conventional MALDI experiment

Some approaches have been developed for the analysis of hydrophobic proteins and peptides by MALDI-MS or ESI-MS. Different solvent compositions have been successfully used to prevent hydrophobic peptides or proteins from aggregating. Schindler and coworkers reported that chloroform/methanol/water mixtures worked well with bacterioopsin in ESI-MS [1]. Neat formic acid was found to give the best result for bacterioopsin (BO) from halobacterium halobium and chloroform/methanol/water (2:5:2,v/v/v) containing 2% acetic acid was found to be the best for analyzing the BO fragments by ESI-MS [2]. The acidic conditions could result in the esterification of the threonine and/or arginine amino acid residues and cleavage of acid-labile peptide bonds [10]. Green-Church and Limbach used nonaqueous solvents such as chloroform and chloroform/methanol solutions to prepare the matrix that they used. Hydrophobic peptides were dissolved in chloroform [3]. This approach is excellent to detect

hydrophobic analytes but not good for the characterization of hydrophilic peptides, thus not one is ideal for hydrophobic/hydrophilic mixture analysis. Recently, Bird *et al.* [4] used a new method, temperature-leap tactic, to maintain the solubility of hydrophobic proteins. All of these solutions don't preserve the protein native conformation and thus are not ideal for our analysis of CcO.

Detergents have also been used to solubilize hydrophobic proteins [5]. They are used to extract membrane proteins. Detergent molecules are amphiphilic molecules. Above their critical micelle concentration (CMC), detergent micelles are formed (Figure 4.7). Their nonpolar tails are hidden in the interior region through hydrophobic interactions and their polar heads are exposed to the water environment. Due to this unique property, detergents can solubilize hydrophobic proteins by forming micelles with them.

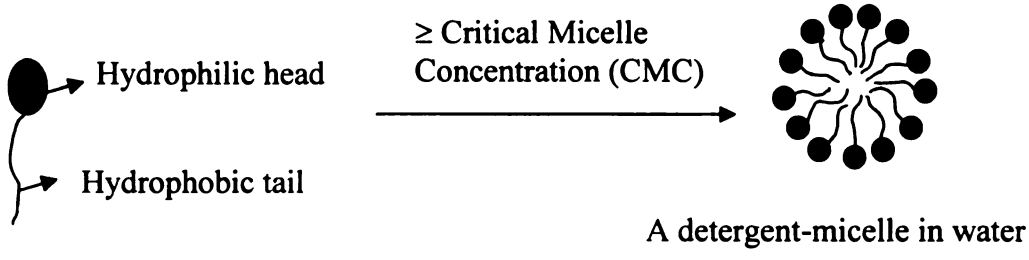


Figure 4.7: The formation of detergent micelles

Detergents can be classified as three types: ionic, non-ionic, and zwitterionic, based on the nature of their hydrophilic head. Ionic detergents contain a head group with a net charge. Non-ionic detergents contain uncharged, hydrophilic head groups. They are better suited for breaking lipid-lipid and lipid-protein interactions than protein-protein

interactions. They are considered non-denaturing. Another advantage of non-ionic detergents is that they can be tolerated at relatively high concentrations in the MALDI MS analysis without degrading the mass spectra. These two advantages make it a possible solution to our problem.

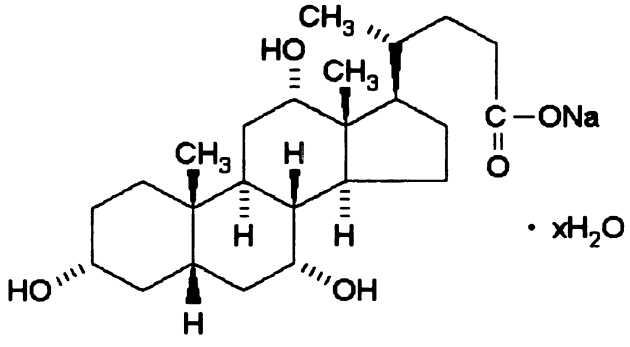
The Modified MALDI TOF MS Experiment for the Detection of Subunits I, II and III

Experimental Section

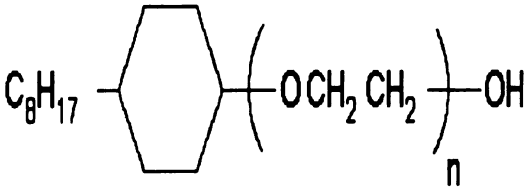
To investigate the effects of different detergents on the detection of the three hydrophobic subunits of CcO in MALDI-TOF-MS, the following detergents were evaluated: sodium cholate, lauryl maltoside, decyl maltoside, polyoxyethylene 23 lauryl ether (brij-35), triton-100, and polyethylene glycol sorbitan monolaurate (tween-20). The structures and physical properties of these detergents are shown in Table 4.4 and Figure 4.8.

Table 4.4: Detergents Evaluated for CcO Analysis Using MALDI MS

Detergent	Detergent Type	M.W.	CMC (mM)
Sodium cholate	Anionic Bile acid	414.6	9-15
Triton-X100	Non-ionic Polyoxyethylene	650	0.25
Tween-20	Non-ionic Polyoxyethylene	1228	0.059
Brij-35	Non-ionic	1200 (avg.)	0.09
Lauryl Maltoside	Non-ionic	510.1	1.8

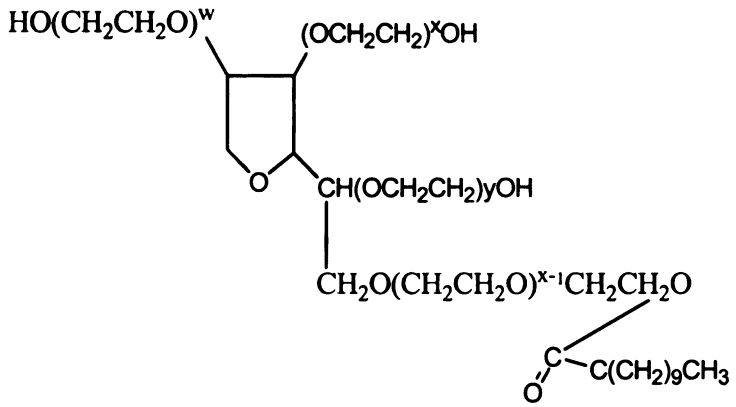


Sodium cholate



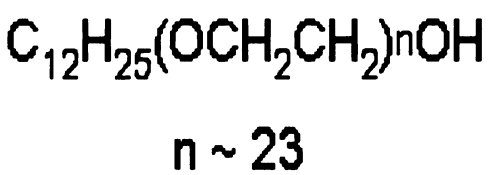
n ~ 9.5

Triton-X100

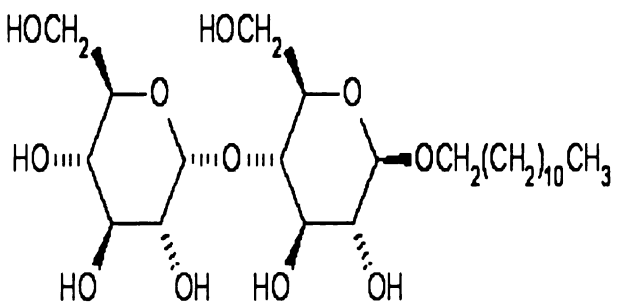


Sum of w+x+y+z=20

Tween-20



Brij-35



Lauryl Maltoside

Figure 4.8: The structure of the detergents evaluated.

The matrices SA, ATT, DHB were used by dissolving them in ACN/water (v:v=1:1) solution. Equal volumes of matrix and protein solutions were deposited on the MALDI sample well. Bovine serum albumin (BSA), purchased from Sigma-Aldrich (St. Louis, MO), was used for calibration.

Linear MALDI mass spectra were recorded on a PerSeptive Biosystems (Framingham, MA) Voyager delayed extraction (DE) time-of-flight (TOF) mass spectrometer with a nitrogen laser (337 nm, 3 ns pulse length). Typically 50 laser shots were averaged for each spectrum. The accelerating voltage was 25 kV, the delayed time was 600 ns, the grid voltage was 90% of the accelerating voltage, and the magnitude of the guide wire voltage was 0.3% of the accelerating voltage. All spectra were obtained in positive mode.

Results and discussion

Different detergents show very different properties that have a very important influence on the protein solubilization and MALDI analysis. The type of detergent used for a specific protein and the amounts are critical for a successful solubilization of the membrane protein [6]. The selection of a proper detergent for a target protein is a trial-and-error procedure. Some studies found that triton X-100 [7] and sodium cholate [8] are effective at solubilizing CcO. But they actually don't influence the detection of subunits I, II and III in MALDI-MS.

Lauryl maltoside (LM) turned out to be the best for the analysis of the hydrophobic subunits of CcO in MALDI-MS. This result is in good agreement with the study of Ferguson-Miller, which showed that lauryl maltoside is the best detergent to fully disperse CcO [9]. Using lauryl maltoside as an additive to the MALDI matrix, subunits I, II and III were successfully detected. Subunit II is easier to be detected since it is relatively hydrophilic. The spectrum is shown in Figure 4.8.

However, a new question was raised: since detergent molecules separate these hydrophobic subunits by forming micelles with them, we would expect to see a peak in the MALDI spectrum representing the detergent micelle bound to each subunit. A typical lauryl maltoside micelle consists of about 140 monomers and weighs around 76,000 Da if there is no protein present in the solution. If a lauryl maltoside micelle is formed around the protein, a larger micelle would be expected to accommodate the protein molecule. The bigger a protein molecule, the larger a detergent micelle would be expected. It was found that the lauryl maltoside micelle around a CcO monomer weighs about 106,000 Da [20]. We can estimate that the lauryl maltoside micelle around each hydrophobic subunit will weight between 76,000 and 106,000 Da. For example, we would expect to see a peak representing subunit III bound to lauryl maltoside (LM), (subunit III) $(LM)_{140\text{-}210}$ in the MALDI spectrum (See Figure 4.9). In fact, it was not observed. Only individual subunits were detected. Why wasn't the subunit-detergent micelle complex detected?

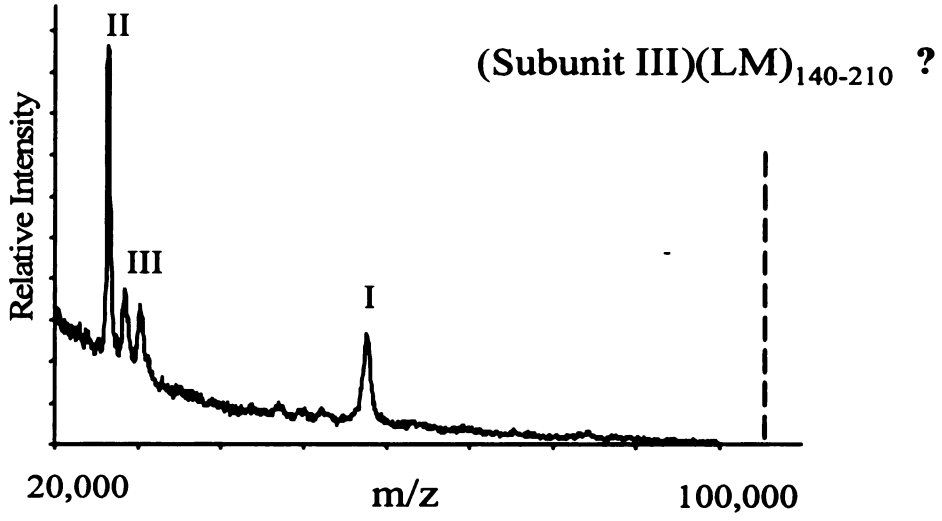


Figure 4.9: Positive-ion linear MALDI-TOF spectrum of cytochrome c oxidase. Subunits I, II and III were detected. ATT was used as matrix. Lauryl maltoside was used as an additive.

Actually, this is another example of the dissociation of a non-covalent complex in MALDI-MS. To illustrate this issue, it needs more detailed study on the mechanism of how the protein is incorporated into the matrix crystal lattice. Beavis and Bridson did a study using *trans*-sinapic acid [11]. Crystals of sinapic acid were produced in a solution containing a specific dye (Coomassie Brilliant Blue G250) to determine which of the crystal faces were responsible for the protein inclusion. The crystals were grown by slow evaporation. X-ray crystallography suggested that the face that is responsible for the interaction with an analyte protein shows great non-polar properties. It lacks free hydroxyl groups for hydrogen bonding. It was concluded that hydrophobic interactions are the primary interactions.

If this mechanism is true, we can explain the dissociation of the detergent-subunit complex in the following way: the hydrophobic tails of detergent molecules bind to the hydrophobic portions of the protein. The resulting micelle no longer has the amphiphilic character. When the micelle comes into contact with the growing crystal surface, the competition between matrix and detergent for the hydrophobic portion of the protein results in the dissociation of the micelle, releasing the protein to the crystal surface. This process is shown in Figure 4.10 1).

Some researchers have questioned Beavis's study. Firstly, their crystals were grown under an equilibrium state, which is quite different from the real MALDI fast growth of crystals [12]. Secondly, the presence of the dye could have an effect on the crystallization. The dye might be specifically binding to hydrophobic faces. As a result, a question was raised: is the incorporation of analytes into matrix crystals a prerequisite for MALDI? The concept of analyte incorporation as a prerequisite was ruled out by the study of Horneffer *et al.* [12] and Glückmann *et al.* [13]; however their work is not widely accepted.

The dissociation of detergent-subunit complexes could also have occurred in the desorption/ionization process since the interaction between them is very weak. The detergent micelle-subunit complexes didn't dissociate when the crystals were formed. The complexes were trapped inside the matrix crystal lattice. Upon the laser irradiation, the detergent molecules fall off from the complex. This process is shown in Figure 4.10 2).

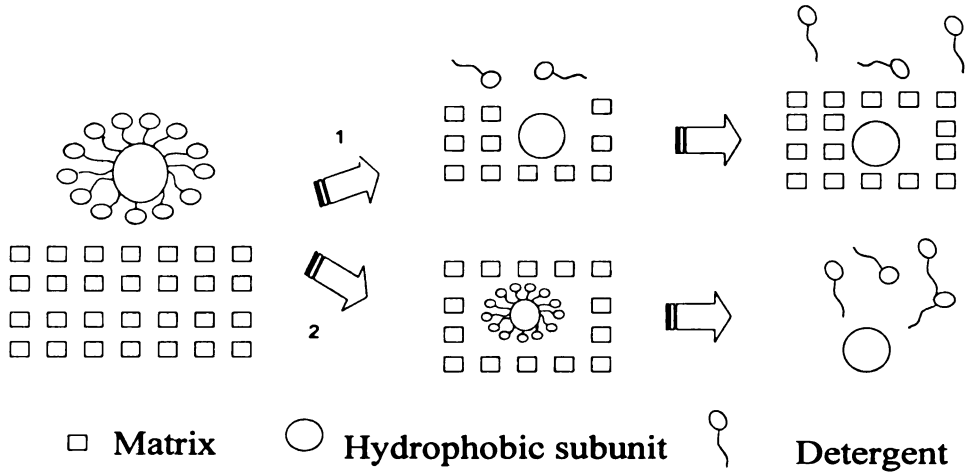


Figure 4.10: The detergent micelle-subunit complexes could fall apart in MALDI MS. 1. the complexes fall apart when they are incorporated into the matrix crystals. 2. The complexes dissociate in the desorption/ionization process.

There are some other factors observed that influence the detection of subunits I, II and III, such as the concentration of detergent and the concentration of the protein.

The spectra obtained using different concentrations of lauryl maltoside solutions are shown in Figure 4.11. The original CcO sample was in a low concentration of detergent solution. When water was used to dilute the protein, it also diluted the detergent and hindered the micelle formation. Theoretically, only above the critical micelle concentration (CMC), can detergent molecules form micelles and solubilize membrane proteins by surrounding them with a hydrophobic cavity. However, it was found in our experiments that even when lauryl maltoside is used, when used at concentrations slightly above CMC, subunits I, II, and III are still not detected. Using SA as the matrix, when the concentration of lauryl maltoside was increased to 10*CMC, subunits I and II were

observed. Subunit III is extremely hydrophobic. It makes up the one of major portions of the enzyme that is embedded in the membrane [20]. It was not detected in the low concentration of detergent. When the detergent concentration was increased to 100* CMC, subunits I, II and III were detected.

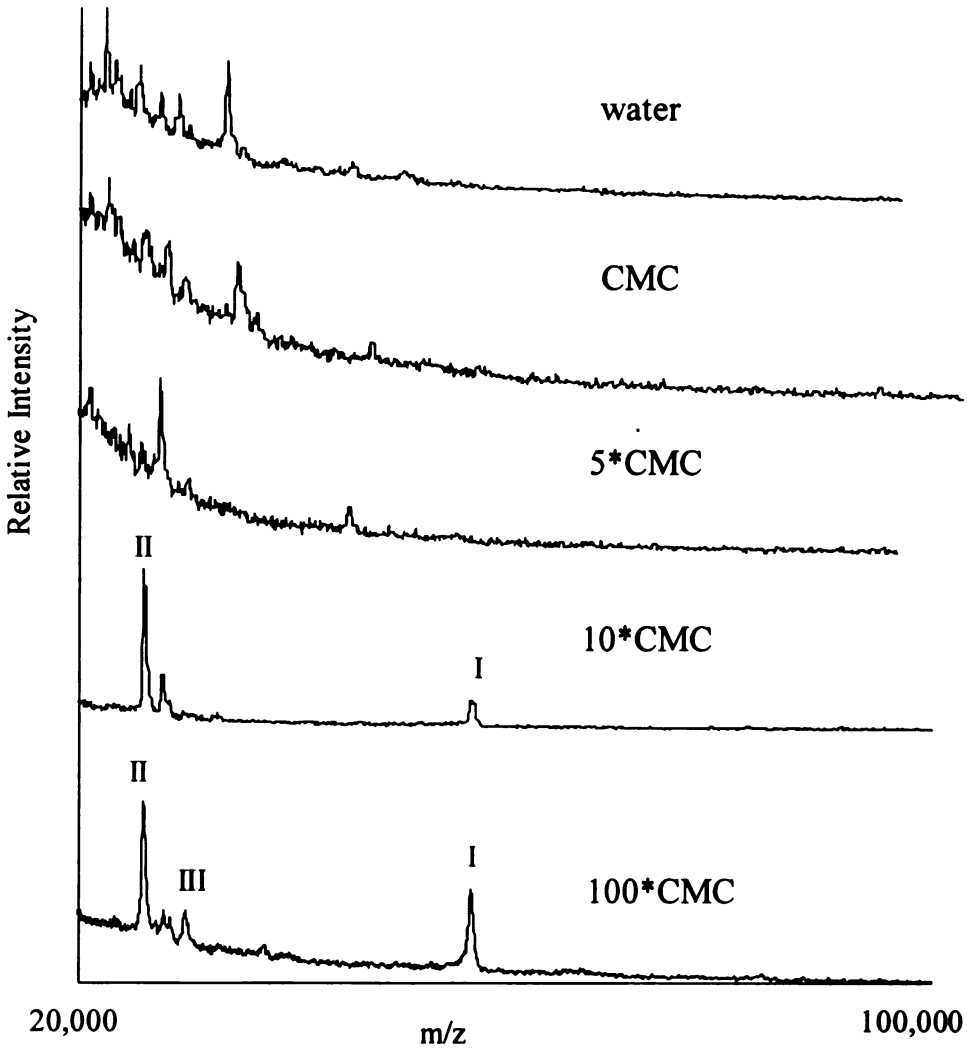


Figure 4.11: Positive ion linear MALDI-TOF spectra of subunits I, II and III of cytochrome c oxidase using different concentration of lauryl maltoside as solvent. SA was used as the matrix. CcO concentration: 1μM

Similar results were obtained when ATT was used as the matrix. The difference is, subunits I, II, and III were detected when the lauryl maltoside concentration is as low as the CMC. Thus, ATT is a better matrix for the detection of the three hydrophobic subunits.

The effect of the concentration of CcO was also investigated. The spectra are shown in Figure 4.12. A solution of 8*CMC lauryl maltoside was used to dilute CcO in all the following experiments. When concentrations of CcO were above 2 μM, subunits I, II, and III were not detected. Instead, some unknown peaks were observed in that mass range. These peaks couldn't be assigned to any of the subunits. They might be resulting from the aggregation of some smaller subunits. When the CcO concentration was about 1 μM, these unknown peaks were not observed and subunits I and II were observed. When the CcO concentration was decreased to 0.5 μM, all subunits I, II and III were detected. It seems that lower CcO concentrations can help detect these hydrophobic subunits at certain detergent concentrations. The reason might be that at lower concentrations the aggregation process occurs more slowly.

Similar results were obtained when ATT was used as the matrix. Those three subunits can be detected when the concentration of CcO was between 5 μM and 0.1 μM.

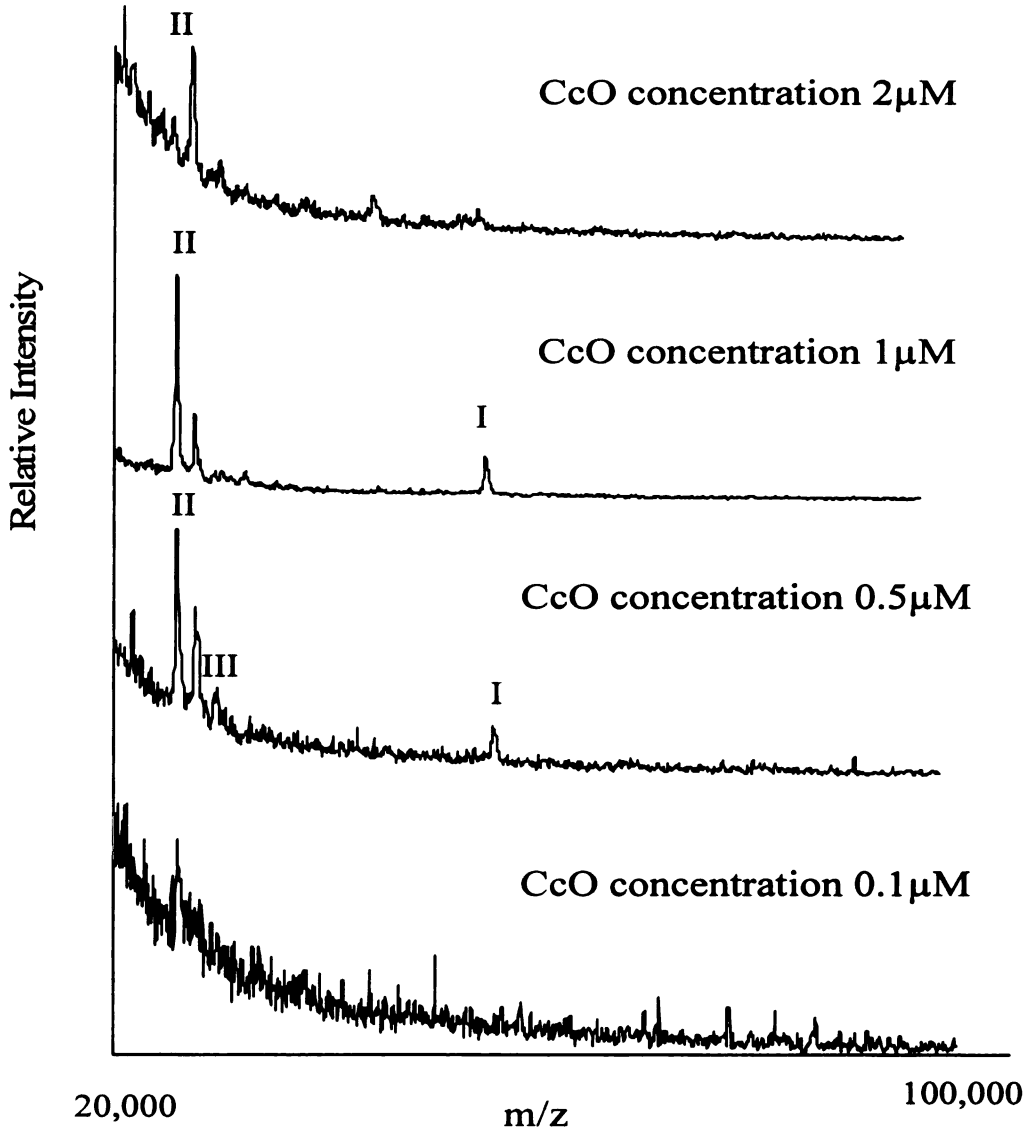


Figure 4.12: Positive ion linear MALDI-TOF spectra of subunits I, II and III of cytochrome c oxidase in different concentration. SA was used as the matrix. The concentration of lauryl maltoside: 8*CMC

Our experiments proved that the detergent concentration and protein concentration are critical variables for the detection of the hydrophobic subunits of CcO using MALDI-TOF-MS. Consider that detergent molecules are present in the solution to help prevent the hydrophobic subunits from aggregating. What really matters is not the absolute concentration of CcO but the ratio of the concentration of detergent to protein. At a certain ratio of [CcO]:[lauryl maltoside], the detergent can fully disperse the hydrophobic subunits without imposing detrimental effects on the MALDI analysis. When the [CcO]:[lauryl maltoside] ratio is too high, hydrophobic subunits are not fully solubilized and may aggregate and not be detected. When the [CcO]:[lauryl maltoside] ratio is too low, too many detergent molecules might interfere the crystallization process and degrade the spectrum. If the absolute concentration of CcO is beyond the sensitivity, those subunits were also not detected.

Conclusion

Cytochrome c oxidase does dissociate in the conventional MALDI experiment. Only the ten nuclear coded subunits could be detected when conventional MALDI conditions were used. Improved experiments were achieved by the use of non-ionic detergents as matrix additives. Among all the detergents evaluated in our experiments, lauryl maltoside is the best one to fully disperse this membrane protein and make all of its subunits accessible to MALDI detection. Some small subunits were detected using ESI-FTMS. They confirmed the results from MALDI-TOF-MS.

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Chapter Five: The Study of Cytochrome C Oxidase Complexes Using MALDI-TOF-MS

Experimental Section

Cytochrome c oxidase sample C (See chapter 4 experimental section) was used in all the experiments. The compounds 2,5-dihydroxybenzoic acid (DHB), 6-aza-2-thiothymine (ATT), sinapinic acid (SA), and alpha-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (St. Louis, MO). The standard solutions of these MALDI matrices were prepared using water. Ammonium acetate, purchased from Sigma-Aldrich (St. Louis, MO), was added to adjust the solution pH. Lauryl maltoside was used as a detergent to solubilize cytochrome c oxidase. Bovine serum albumin (BSA), purchased from Sigma-Aldrich (St. Louis, MO), was used for calibration.

Linear MALDI mass spectra were recorded on a PerSeptive Biosystems (Framingham, MA) Voyager delayed extraction (DE) time-of-flight (TOF) mass spectrometer with a nitrogen laser (337 nm, 3 ns pulse length). Typically, 50 laser shots were averaged for each spectrum. The accelerating voltage was 25 kV, the delayed time was 800 ns, the grid voltage was 90% of the accelerating voltage, and the magnitude of the guide wire voltage was 0.3% of the accelerating voltage.

Results and discussion

The first problem encountered in the analysis of cytochrome c oxidase (CcO) complexes using MALDI-TOF-MS is the calibration of the high mass range. The molecular weight of CcO is above 200,000 Da. The calibration species usually used for the high mass

range in MALDI MS are: bovine serum albumin (M.W. 66,431), cytochrome c (M.W. 12,361) and Immunoglobulin G (IgG) (M.W. 144, 391). None of these are appropriate to calibrate spectra of cytochrome c oxidase complexes.

It was observed that, in the positive MALDI mass spectrum of insulin, the insulin monomer forms the base peak, but a multitude of oligomers is seen as well [1]. When the analyte concentration is high enough, non-specific aggregation can occur. This can be a solution to achieving the high molecular weight calibration. Figure 5.1 shows the BSA dimer and trimer detected in MALDI-TOF-MS, which can be used to calibrate the spectrum for m/z values as high as that for the cytochrome c oxidase monomer peak.

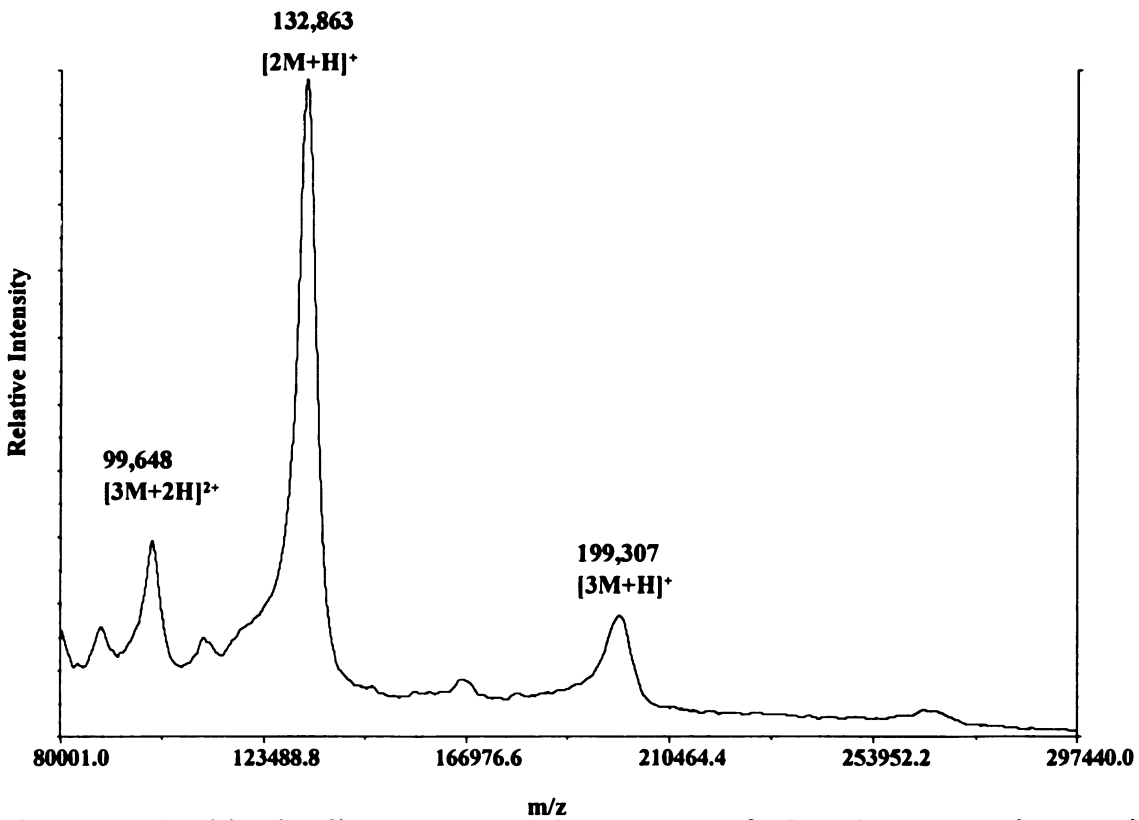

Figure 5.1: Positive-ion linear MALDI-TOF spectrum of BSA. SA was used as matrix. Concentration of BSA: 50 μM.

In the conventional MALDI analysis of CcO, no ions above 100,000 Da were detected. Cytochrome c oxidase does dissociate and only individual subunits were observed. The MALDI experimental conditions could have possibly disturbed the native structure of the protein. Organic solvents, matrix, and low pH could weaken the non-covalent interactions. In order to eliminate these detrimental effects, variations in the experimental conditions were tested.

As mentioned in chapter one, the matrix 6-aza-thiothymine (ATT) is close to neutral in pH and has found success for the detection of duplex-DNA, protein and peptide complexes. The first advantage of the use of ATT is that it does not create an acidic environment. The denaturation of CcO usually occurs at a pH lower than 5 [4]. Another advantage of ATT is that it is readily soluble in aqueous solution.

Different combinations of water and ACN were tested. When pure water was used as the solvent, several complexes were detected. The results become more reproducible by the addition of ammonium acetate. It is not well known why the addition of ammonium acetate helps the detection of cytochrome c oxidase complexes in MALDI MS. It might be due to the pH (pH is about 6.5), better cocrystallization, or facilitation in the desorption/ionization process. Other ammonium salts were also evaluated, such as ammonium citrate and ammonium bicarbonate. They don't provide much improvement.

Since cytochrome c oxidase is a membrane protein, it is found that some lipids are still attached to the protein after it is isolated. Nine phospholipids, five phosphatidyl

ethanolamines (PE), three phosphatidyl glycerols (PG) and one phosphatidyl choline (PC) have been clearly found in cytochrome c oxidase from bovine heart. In addition to the lipids mentioned above, cardiolipin has long been known as one of the phospholipids that cannot be removed without the loss of the enzyme activity. Five cardiolipins were found to be bound to the beef heart enzyme [6].

In an early study of Ferguson-Miller *et al.* [5], an apparent molecular weight, 300,000-350,000 resulting from gel filtration, was obtained for CcO from bovine heart in the presence of lauryl maltoside. In order to identify this species as a monomer or a dimer, the sedimentation equilibrium analysis in solvents of different densities was performed. It gave the molecular weight of both the protein moiety and the protein-detergent complex. The difference between them provided an estimate of the amount of associated detergent. The mass of lauryl maltoside bound to the beef heart oxidase is about 106,000 amu.

Table 5.1 shows the main species and their approximate molecular weights in CcO from bovine heart.

Table 5.1: The Main Species in Cytochrome c Oxidase Enzyme from Bovine Heart and Their Approximate Molecular Weights

Species	Approximate molecular weight
Cardiolipin	1500
5 PE + 3 PG + 1 PC	6,750
Cytochrome c oxidase monomer	205,000
Heme	852
Lauryl maltoside micelle around CcO	106,000 + 20,000
CcO+5CL+5PE+3PG+1PC+2Heme	326,954

Using ATT as matrix, ammonium acetate and laury maltoside as matrix additives, some peaks representing cytochrome c oxidase complexes are successfully detected in MALDI-TOF-MS.

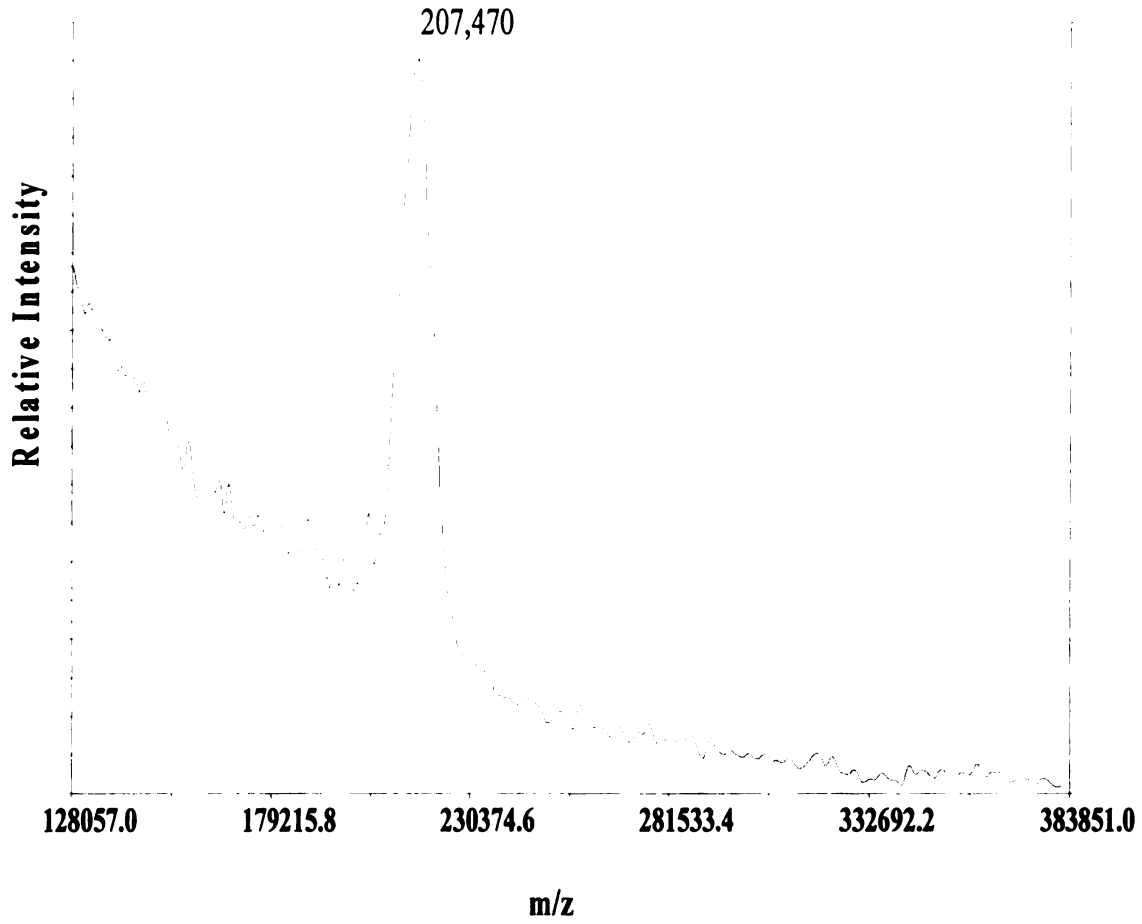

Figure 5.2: Positive-ion linear MALDI-TOF spectrum of CcO complexes. ATT (in 60 mM NH_4OAc) was used as matrix. Concentration of CcO: 3 μM.

The peak shown in Figure 5.2 could possibly represent the monomer of cytochrome c oxidase with thirteen subunits in it. The expected molecular weight is 204,751 while the m/z value from the MALDI spectrum is 207, 470, which is 1% higher than the expected value. It is possible that some phospholipids are bound to the monomer and make the m/z value higher than the actual molecular weight of CcO monomer.

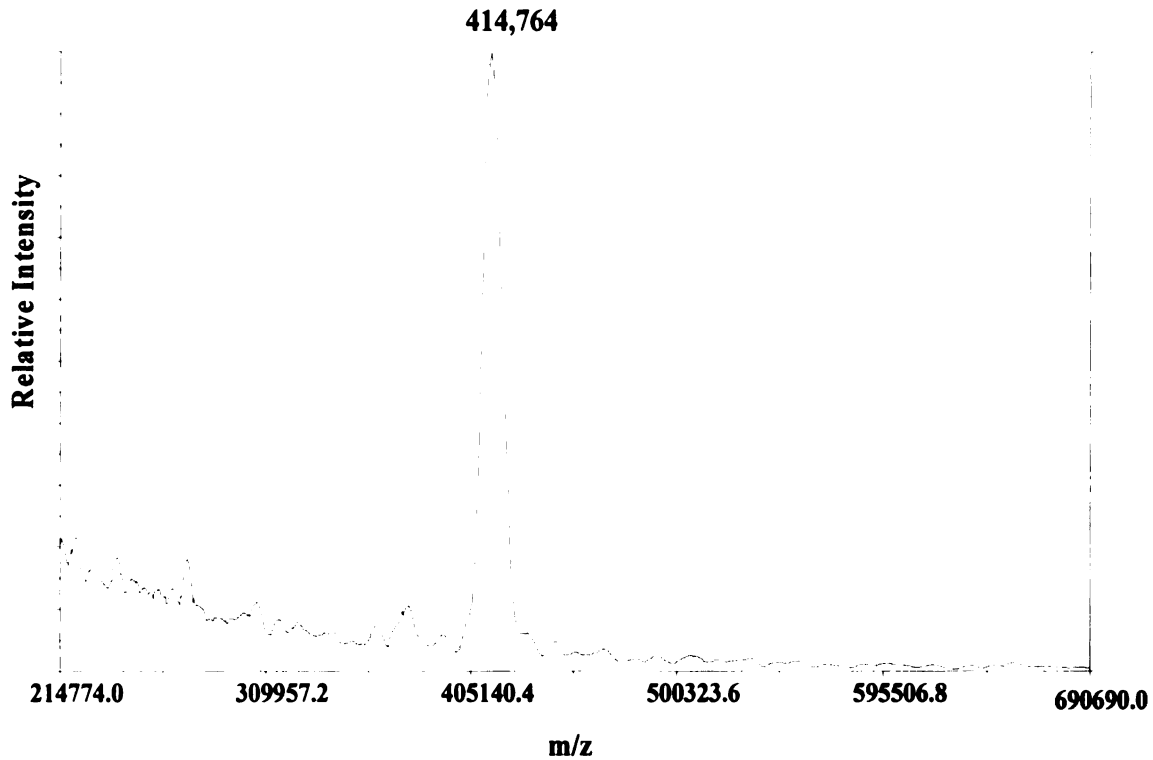

Figure 5.3: Positive-ion linear MALDI-TOF spectrum of CcO complexes. ATT (in 60 mM NH_4OAc) was used as matrix. Concentration of CcO: 3 μM.

The peak shown in Figure 5.3 could represent the dimer of cytochrome c oxidase ((subunits I-XIII)$_2$). The expected molecular weight is 409,502, while the m/z detected in MALDI-MS is 414,764. The m/z value for the peak in MALDI mass spectrum is 5,264 higher than the expected value. It could have seven phospholipids or three cardiolipins attached to the protein.

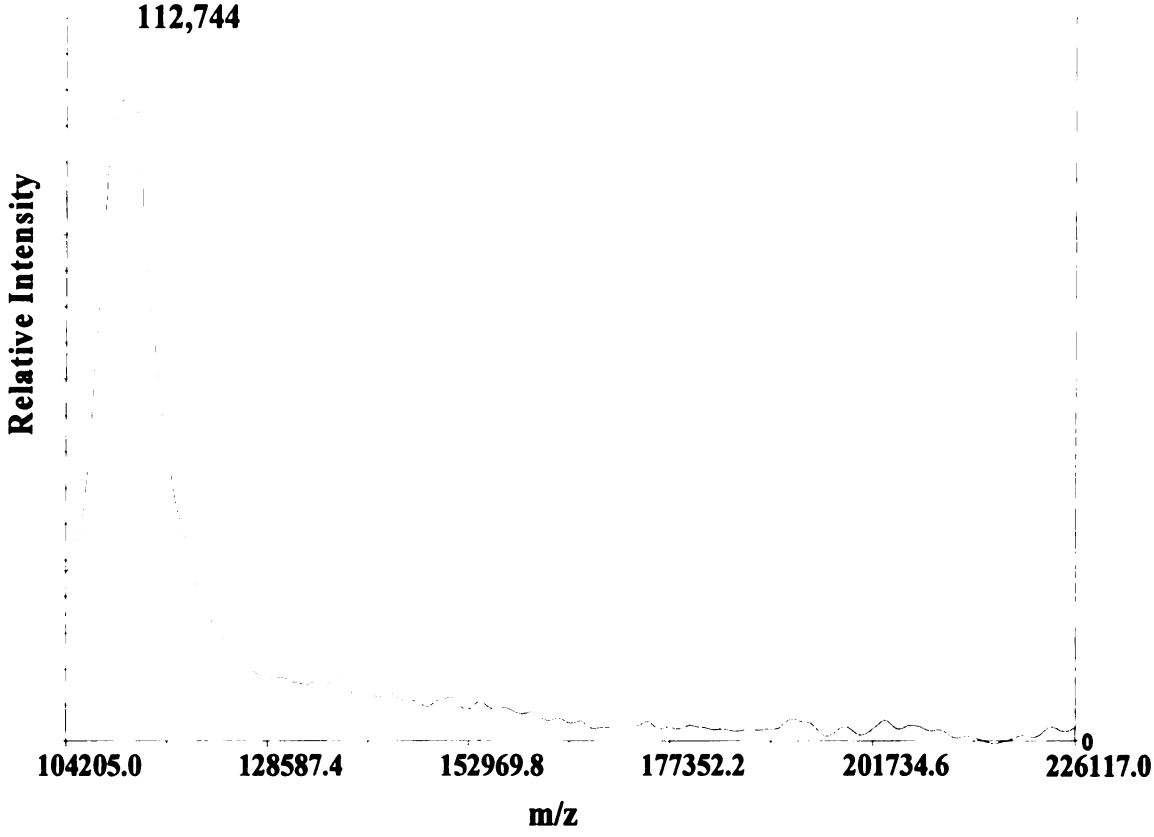

Figure 5.4: Positive-ion linear MALDI-TOF spectrum of CcO complexes. ATT (in 60 mM NH_4OAc) was used as matrix. Concentration of CcO: 3 μM.

The peak shown in Figure 5.4 could represent the complex of subunit I binding to subunits II and III. Subunits I, II and III are the three core subunits in cytochrome c oxidase. They are located mainly in the transmembrane space. Subunit I is directly interacting with subunits II and III. The hydrophobic interactions between them are stronger than the interactions among the other smaller subunits. The partial dissociation of the enzyme leads to the smaller subunits falling off while the three largest ones still remain together. The expected molecular weight for subunits I + II +III is 112,744 while the m/z detected in MALDI-MS is 113,500. The difference is 756 Da.

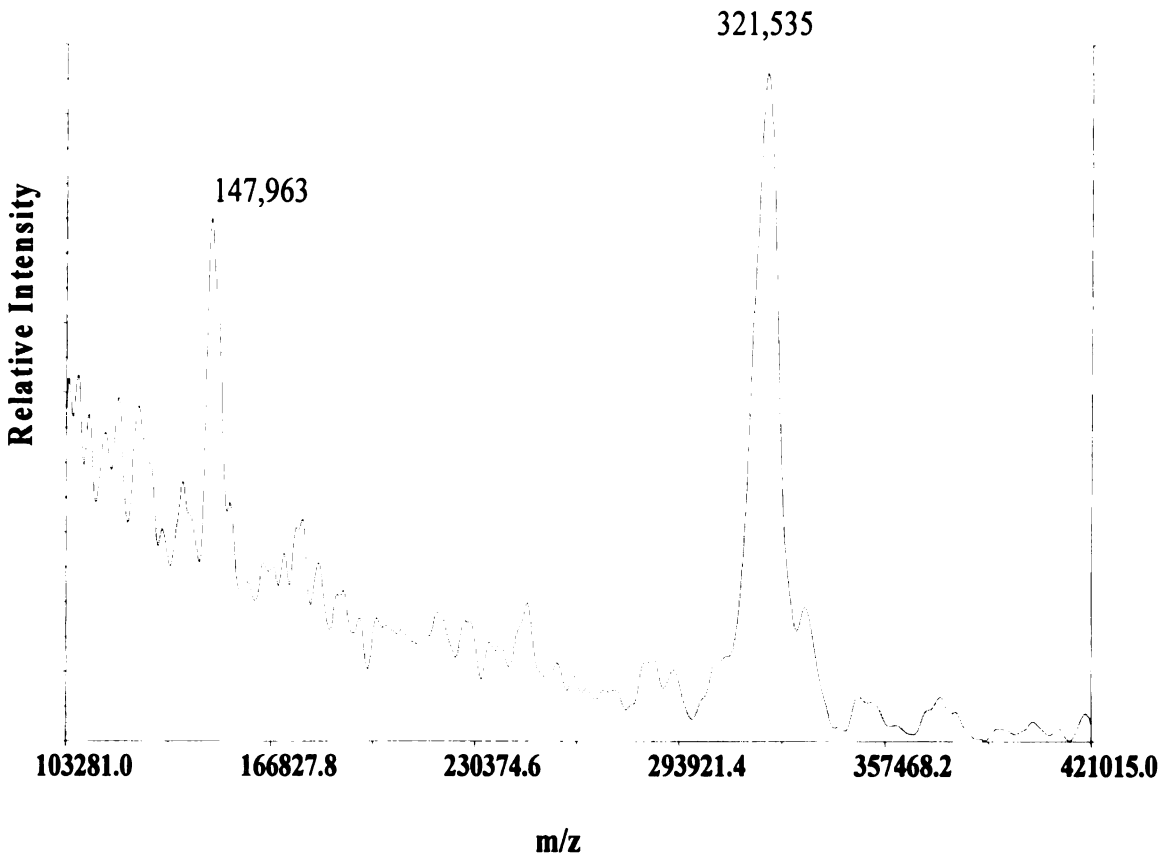

Figure 5.5: Positive-ion linear MALDI-TOF spectrum of CcO complexes. ATT (in 60 mM NH_4OAc) was used as matrix. Concentration of CcO: 3 μM.

The peak shown in Figure 5.5 at m/z 147,963 could represent the subunit III-depleted enzyme. To study the biological function of subunit III, subunit III has been removed by the use of detergent, incubation, ion exchange or affinity chromatography [1]. These methods yield preparations containing less than 15% of the normal complement of subunit III, but also cause the removal of several of the smaller peptides (subunits VI, VII and VIII) [1]. This will give a molecular weight of 145,000 Da (205,000-29,918-10,670-10,067-9436).

The other peak in Figure 5.5 at m/z 321,535 could represent the detergent micelle binding to the monomer. It was found that the lauryl maltoside micelle around beef heart cytochrome c oxidase has a molecular weight of approximately 106,000 as determined by the sedimentation equilibrium method [2]. That will give a molecular weight of 327,000 while the mass detected in MALDI MS is 321,535. The difference is about 5,465. This could mean that there are some phospholipids bound to the protein.

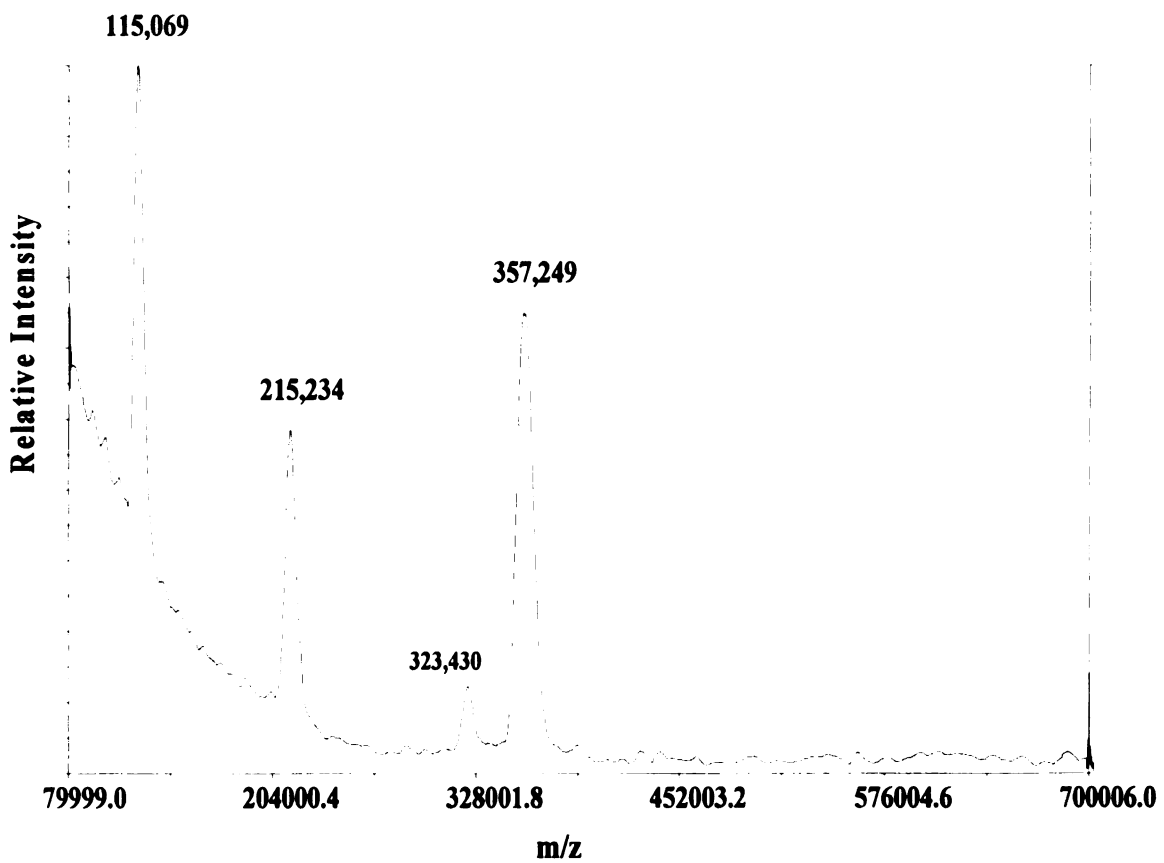

Figure 5.6: Positive-ion linear MALDI-TOF spectrum of CcO complexes. ATT (in 60 mM NH_4OAc) was used as matrix. Concentration of CcO: 3 μM.

The peak shown in Figure 5.6 has m/z value of 215,234, which is 10,000 Da higher than the molecular weight of CcO monomer. This peak could represent five cardiolipins and three phospholipids attached to the monomer. It could also represent fourteen phospholipids and two cardiolipin lipids bound to the monomer.

The other peak in Figure 5.6 at m/z 357,249 could present the subunit III-depleted dimer. The calculated molecular weight is 350,000 (410,000-29,932-10,670-10,067-9436). If it has five cardiolipins attached, this will give a molecular weight of about 357,500(350,000+7,500).

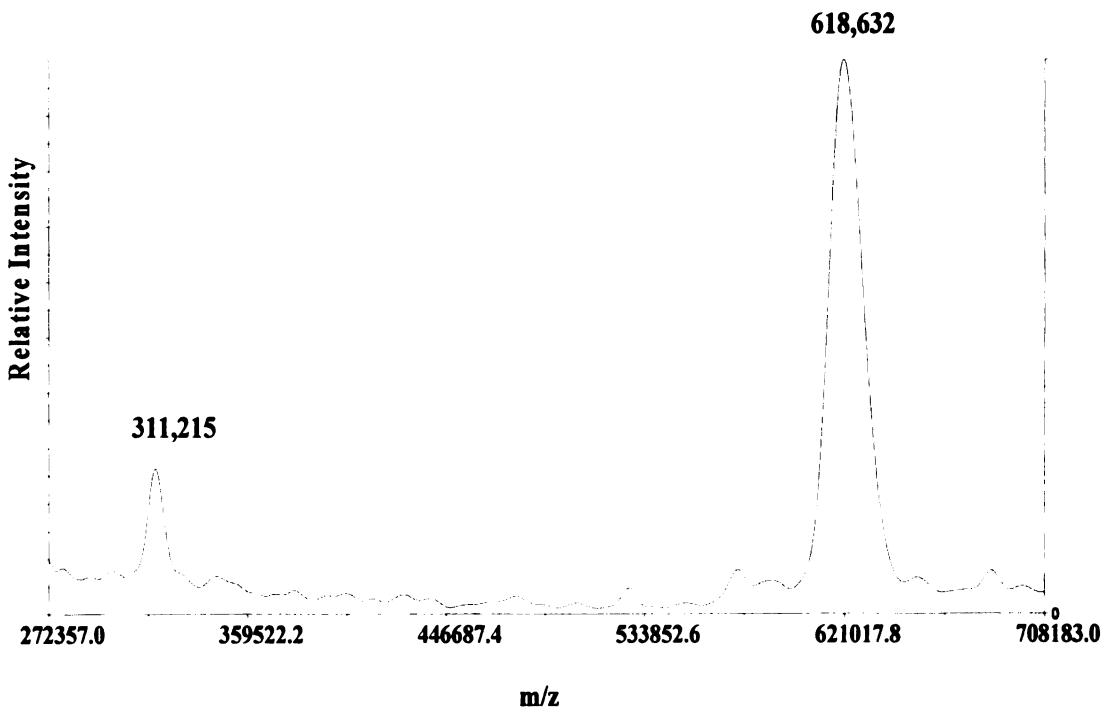

Figure 5.7: Positive-ion linear MALDI-TOF spectrum of CcO complexes. ATT (in 60 mM NH_4OAc) was used as matrix. Concentration of CcO: 3 μM.

The peak shown in Figure 5.7 at m/z 311,215 could represent the lauryl maltoside micelle binding to the cytochrome c oxidase monomer. The calculated molecular weight is 311,000 (205,000+106,000). That matches the mass detected in MALDI MS very well.

The other peak in Figure 5.7 at m/z 618,632 could represent the cytochrome c oxidase trimer. The expected molecular weight is 615,000.

Actually all these peaks representing CcO complexes detected using MADLI MS were from a heterogeneous target. MALDI has a notorious reputation for its variability. Although the analyte and matrix mix very well in the solution, upon the solvent evaporation, they form heterogenous sample crystals. The radius of the sample well is about 1 mm, which is much smaller than the size of the laser beam. When the laser irradiates different sample areas, different MALDI spectra will be obtained, with varying signal intensity and resolution. In our study of the cytochrome c oxidase complexes, many different forms of the enzyme have been detected: the intact monomer and dimer, some phospholipids binding to the protein, the detergent micelle binding to the protein, and the protein losing some smaller subunits. These complexes were detected in different MALDI sample spots and made it hard to get consistent results. Why are different complexes formed in different areas of the sample plate? Consider that the MALDI crystallization of the matrix and analyte mixtures occurs in a very short time, usually one to three minutes. The fast solvent evaporation has great influence on the crystal formation. In some areas of the surface, the solvent evaporates very fast and the concentrations of analyte molecules and matrix are very high. They get saturated on the

surface even they are not saturated in the bulk solution yet. The crystals will be formed on the surface first. The crystals formed at the beginning of the crystallization process are very difference from those that are formed at the end of the crystallizatio.

Another problem is the low resolution at the high m/z values. Figure 5.8 shows the peak representing the cytochrome c oxidase monomer detected in MALDI MS.

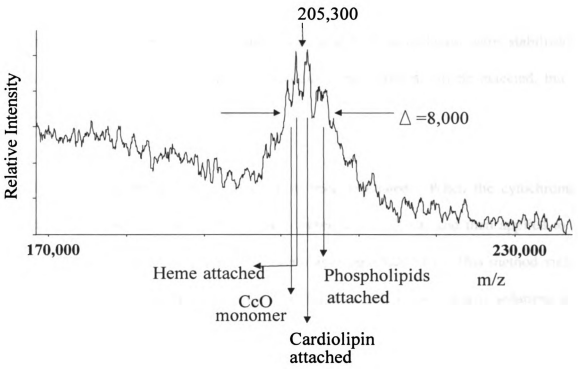

Figure 5.8: Low resolution at high m/z values

The half width of this peak is about 8,000 Da. The resolution is about 26. The poor resolution makes it very hard to tell the actual form of the enzyme detected. The molecular weight of each phospholipid is about 750 Da. We can not distinguish the

monomer from monomer-phospholipids complexes based on this peak. Similarly, there is no way to determine whether the heme is still bonded to the protein or not. We lack information to differentiate the different forms of the enzyme due to this wide peak.

The use of the matrix ATT in an aqueous ammonium acetate solution successfully stabilized the cytochrome c oxidase complexes for the MALDI analysis. Our experimental results suggest that organic solvents and acids are the primary reasons for the dissociation of the cytochrome c oxidase complexes. Meanwhile we found evidence from the lower mass range to prove that some CcO complexes were stabilized in MALDI. Under this condition, the individual subunit could still be detected, but the intensity has been dramatically decreased.

Different sample preparation methods have been evaluated. When the cytochrome c oxidase solution and matrix solution were mixed in a vial first and then applied to the sample plate, it could increase the sensitivity and reproducibility. This method yielded more homogeneous crystals than if the cytochrome c oxidase and matrix solutions were sequentially deposited onto the sample well.

It was reported by our lab that several peaks representing complexes of cytochrome c oxidase subunits from *R. Sphaeroides* were detected when sucrose was added to the cytochrome c oxidase solution [3]. The presence of sucrose can stabilize proteins in solution. Unfortunately, sucrose doesn't help stabilize bovine heart enzyme very well.

Conclusion

Several cytochrome c oxidase complexes from bovine heart have been detected using appropriate matrix, solvent, and pH. Our experiments suggest that the organic solvents and low pH, rather than the desorption/ionization process itself, are the primary reasons that limit the detection of the cytochrome c oxidase complexes using MALDI MS. Careful attention to the effect of solvent compositions and pH will improve the capability of detecting weakly bound non-covalent complexes using MALDI MS.

References

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Chapter Six: Conclusions and Future Work

While the analysis of membrane proteins by mass spectrometry has proven to be difficult, the multi-component membrane protein cytochrome c oxidase from bovine heart was successfully detected using MALDI MS. To prevent the hydrophobic subunits from aggregating, the detergent lauryl maltoside was used. By selecting the right detergent and appropriate concentration, all of the 13 subunits have been observed and their molecular weights were determined by MALDI MS and some of them were detected by ESI FTMS. Another challenge is that the enzyme dissociates in the conventional MALDI experiment. In this work, cytochrome c oxidase was stabilized by the use of a neutral matrix and aqueous solvent. As a consequence, some intact complexes were detected by MALDI MS.

This work is focused on the protein part of this enzyme. However, when analyzing membrane proteins, in addition to the protein part, it is also essential to characterize the lipids present. Lipids are important to stabilize the protein and maintain the activity of the protein. Lipids play a significant role in the structure of the enzyme and should be considered as a part of it. Many phospholipids are found to be bound to the protein after the isolation and purification of bovine heart cytochrome c oxidase. Some proof can be found in the analysis of the cytochrome c oxidase complexes. Peaks with m/z values between the molecular weigh of the CcO monomer and dimer could be assigned to the species that have phospholipids attached to them. However, this alone doesn't provide information on what types of lipids are present and what their structures are. More direct

information can be obtained from the low mass range of the MALDI mass spectrum. The lipids have molecular weights from 700 Da to 1,500 Da. However there are some problems we should realize. Lipids are amphiphilic molecules and not soluble in aqueous solutions, which are usually used in the MALDI experiment. The addition of detergent could be one of the solutions. But if detergent is used to help the dissolution, the analysis using MALDI is possibly complicated by the presence of matrix and the detergent.

MALDI MS not only can provide molecular information, it can also determine the type of lipids present and further elucidate their structure by the performance of post-source decay (PSD) experiments.

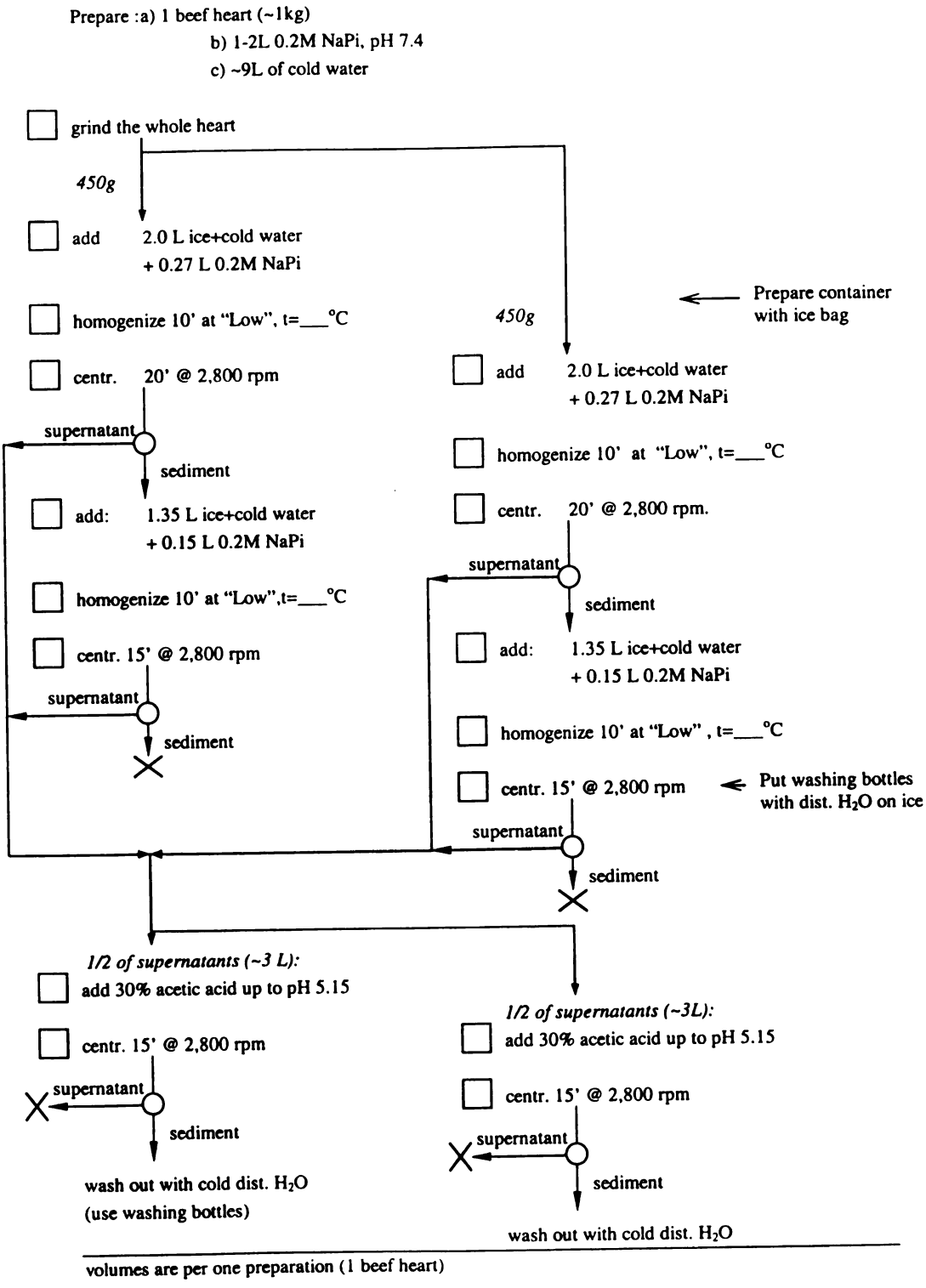
The purification process usually strips some lipids out of the enzyme. As a consequence, different amount of lipids will be present in different stages of purification. Mass spectrometry will be a quick way to determine the types of lipids present. The relative intensities of the peaks can help determine the amount of lipids present. Monitoring the amount and type of lipids present in the enzyme at various levels of purification will be a new application of mass spectrometry.

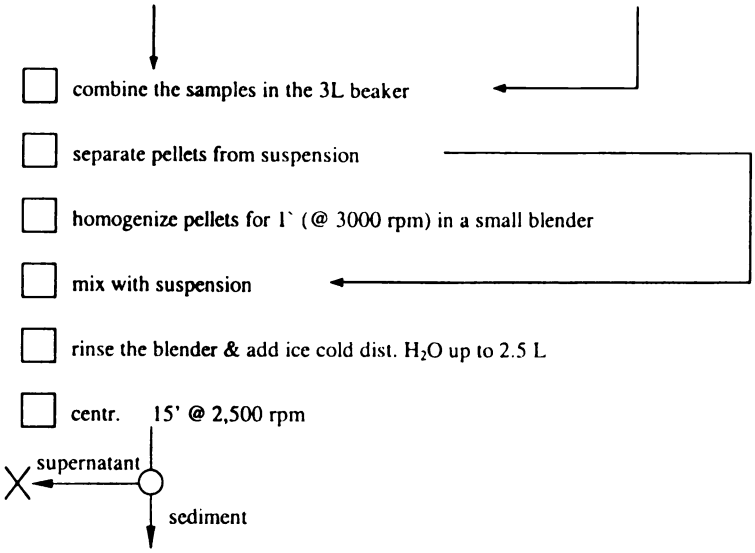
These are all the things to be considered, and additional work needs to be done to continue the study of bovine cytochrome c oxidase.

Appendix

Appendix: Cytochrome c Oxidase purification

After Yoshikawa *et al.* with minor modifications





Prepare Na cholate solution for the second day:

for 20% solution		for 30 % solution
75 mL 0.2 M NaPi, pH 7.4 or	75 mL 0.2 M NaPi, pH 7.4..or	50 mL 0.2 M NaPi
+2.94 g NaOH	+31.6g Na cholate	+ 31.6g Na cholate
+30g cholic acid		
add water up to 150 ml		water up to 100 ml

volumes are per one preparation (1 beef heart)

Second morning:

(if two hearts are used, keep them separately)

☐ ☐ adjust pH from ____ to 7.4

☐ ☐ add Na cholate to final concentration of 3.2 % (86.4 mL of 20% Na cholate into 454 ml of sample)

☐ ☐ add $(NH_4)_2SO_3$: 0-33% or 19.6 g/100 mL (5.4 dL x 19.6 = 105.84 g)

! maintain pH 7.3 - 7.4 using 1N NaOH !

☐ ☐ stir 30' on ice ← ☐ Prepare 200 mL/heart of 2.0% and 500 mL/heart of 0.5% cholate Na

☐ Prepare dialysis buffer (4L/heart)

☐ Prepare dialysis membrane (3x?? cm/heart)

☐ ☐ centr. 20' @ 11,000 rpm

supernatant

sediment

☐ ☐ measure volume (V=____ml) (V=____ml)

☐ ☐ add $(NH_4)_2SO_3$: 33-50% or 10.7 g/100 mL (____g) (____g)

☐ ☐ centr. 25' @ 11,000 rpm

supernatant

sediment

☐ ☐ add 0.5% cholate, final volume 150 mL

resuspend pellets using glass rod

☐ ☐ dialysis 90' against 4 L/heart of 0.04 M NaPi, pH 7.4

☐ ☐ centr. 30' @ 45,000 rpm

supernatant

sediment

☐ ☐ add 2.0% cholate, final volume 200 mL

break pellets with glass rod & homogenize in glass homogenizer

☐ ☐ add $(NH_4)_2SO_3$: 0-25% or 14.4 g/100 mL (28.8g)

monitor pH 7.3-7.4

☐ ☐ incubate 20' on ice without stirring

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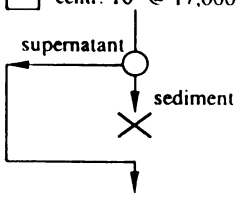
volumes are per one preparation (1 beef heart)

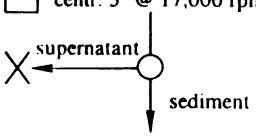
☐ ☐ centr. 10' @ 17,000 rpm



☐ ☐ measure volume (V=____ml) (V=____ml)

☐ ☐ add $(NH_4)_2SO_3$: 25-45% or 12.5 g/100 mL (____g)(____g)

☐ ☐ centr. 5' @ 17,000 rpm

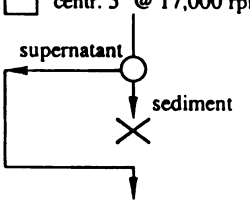


☐ ☐ add 0.5% cholate, final volume 200 mL

☐ ☐ add $(NH_4)_2SO_3$: 0-25% or 14.4 g/100 mL (28.8g)
monitor pH 7.3-7.4

☐ ☐ incubate on ice without 20' stirring

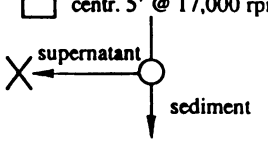
☐ ☐ centr. 5' @ 17,000 rpm



☐ ☐ measure volume (V=____ml) (V=____ml)

☐ ☐ add $(NH_4)_2SO_3$: 25-40% or 9.3 g/100 mL (____g)(____g)

☐ ☐ centr. 5' @ 17,000 rpm



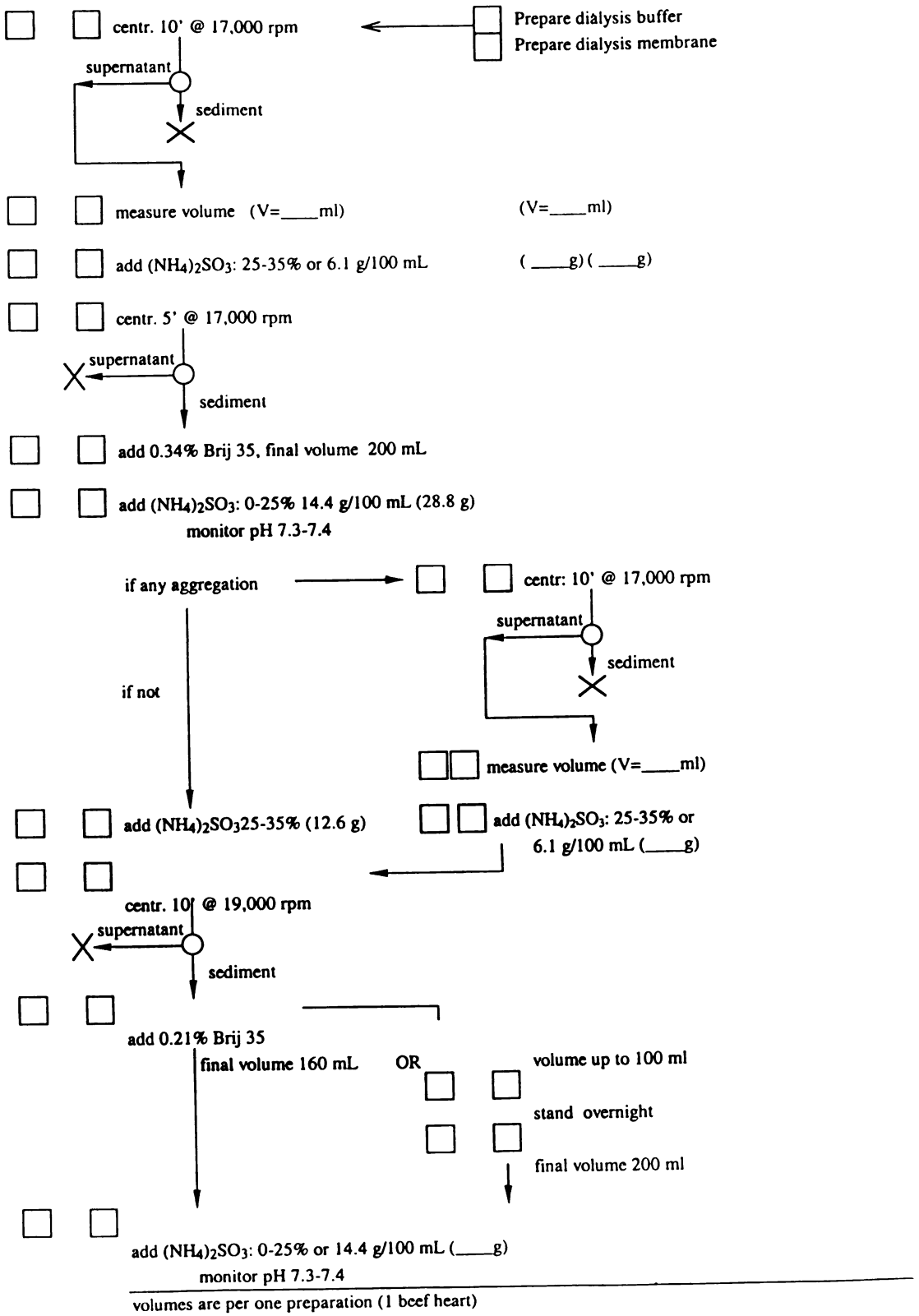
☐ ☐ add 0.5% cholate, final volume 130 mL

☐ ☐ add $(NH_4)_2SO_3$: 0-25% or 14.4 g/100 mL (18.72 g)

☐ ☐ incubate 20' on ice without stirring

☐ Make Brij 35 solutions:
☐ 12 mL/heart 10% Brij 35
200 mL/heart 0.34%
6.8 mL 10% Brij 35
93.2 mL H_2O
100 mL 0.2 M NaPi
☐ 200 mL/heart 0.21%
4.2 mL 10% Brij 35
95.8 mL H_2O
100 mL 0.2 M NaPi

volumes are per one preparation (1 beef heart)


centr. 10' @ 17,000 rpm
Prepare dialysis buffer
Prepare dialysis membrane
supernatant
sediment
measure volume (V=____ml)
(V=____ml)
add $(NH_4)_2SO_3$: 25-35% or 6.1 g/100 mL
(____g) (____g)
centr. 5' @ 17,000 rpm
supernatant
sediment
add 0.34% Brij 35, final volume 200 mL
add $(NH_4)_2SO_3$: 0-25% 14.4 g/100 mL (28.8 g)
monitor pH 7.3-7.4
if any aggregation
centr: 10' @ 17,000 rpm
supernatant
sediment
if not
measure volume (V=____ml)
add $(NH_4)_2SO_3$25-35% (12.6 g)
add $(NH_4)_2SO_3$: 25-35% or 6.1 g/100 mL (____g)
centr. 10' @ 19,000 rpm
supernatant
sediment
add 0.21% Brij 35
final volume 160 mL
OR
volume up to 100 ml
stand overnight
final volume 200 ml
add $(NH_4)_2SO_3$: 0-25% or 14.4 g/100 mL (____g)
monitor pH 7.3-7.4
volumes are per one preparation (1 beef heart)