As I mentioned at the awards ceremony a little while ago, the work for which I have received the Keio Medical Award is not something that I have been able to accomplish alone; it has involved the cooperation of many scientists. Dr. Tsukihara is Professor of the Institute for Protein Research, Osaka University, and specializes in X-ray crystallography. Dr. Kitagawa and Dr. Ogura, who is here today, work with techniques such as resonance Raman spectroscopy. Dr. Itoh works in my laboratory, mainly on the crystallization of cytochrome oxidase. In addition, we of course need money for our research, and our supporters in this regard have been Core Research for Evolutional Science and Technology (CREST) and New Energy and Industrial Technology Development Organization (NEDO).

On a personal note, I have written here that the research environment is extremely important to me. This is the tenth year since the establishment of the Department of Life Science at the Himeji Institute of Technology, and we are fortunate that a very energetic group of researchers has gathered there. The atmosphere we have is indescribably free and non-restrictive. It is difficult to find words that express how very important the atmosphere at the Himeji Institute of Technology has been to me in carrying out my research.

I have also been fortunate to have had outstanding teachers, and I wish to mention, at first, Dr. Kazuo Okunuki, under whom I studied in graduate school. Unfortunately, he passed away this last May, at the age of ninety-one. Thinking back, I realize that to this day my research has been an ongoing investigation of the theme that was assigned to me as a graduate student in Dr. Okunuki’s laboratory. I feel that Dr. Okunuki was important in teaching me what scholarship is all about. The other teacher I wish to mention taught me the importance of chemistry in the study of cytochrome oxidase. I am talking now about Prof. Winslow Caughey, who is currently Professor Emeritus at Colorado State University. In any case, these are the two men I consider to have been my most important mentors.

I wish now to talk about my research. Since we seem to have in the audience today people who are not specialists in the fields of medical chemistry and biochemistry, I will first present a brief introduction on cytochrome oxidase.

As you all know, living organisms eat food and burn it using oxygen, producing carbon dioxide and water as byproducts. The energy that is liberated during this process is used to form ATP from ADP and inorganic phosphate. Since energy is needed to form ATP from ADP, energy is in turn released when ATP returns to inorganic phosphate and ADP. ATP is an extremely important product. Energy in various forms is required for an organism to live, and the energy used in most reactions and necessary physiological processes is supplied almost entirely by ATP. For this reason, ATP is sometimes referred to as the “energy currency.”

The combustion of food with oxygen, however, is slightly different from a car burning gasoline in order to run. The most important difference is that no heat is generated when food is burned with oxygen. Energy is liberated without generating heat.

As I just said, the way an organism uses oxygen during this process, for example, how it uses oxygen to burn glucose, one of the main sources of energy, differs from the way a car burns gasoline. An organism uses a variety of roundabout methods to obtain energy without generating heat. This is one such process, where combustion is achieved not through a direct reaction with oxygen but by first adding water to produce CO₂.
Then, the hydrogen atoms are removed. All the energy is in these hydrogen atoms, and the hydrogen atoms react with oxygen to produce water. Using the energy that is thus released, ADP is converted to ATP, as I said earlier. Cytochrome oxidase, the subject of my research, is involved in this process.

I keep mentioning energy, but what form does this energy take? Cytochrome oxidase forms water from hydrogen atoms and oxygen. Energy is released when water is formed, and, using this energy, cytochrome oxidase transports protons. Cytochrome oxidase is found embedded in the inner mitochondrial membrane of the cell. One property of this inner mitochondrial membrane is its non-permeability to ions, including the proton. Therefore, cytochrome oxidase transports protons through the protein, producing different concentrations of these ions on either side of the membrane. The transport of protons in this way is called active transport, or the proton pump.

Energy is needed to transport protons from one side of the inner mitochondrial membrane to the other. On the other hand, the presence of a slight opening here would cause protons to flow naturally from the side of higher concentration to that of lower concentration. The passage of protons in this direction creates a reverse situation in which energy is needed to transport the ions against the gradient; thus energy is released. This energy is used to produce ATP from ADP and inorganic phosphate.

ATP synthase and cytochrome oxidase are considered the two most important enzymes in the mechanism of respiration.

Cytochrome oxidase works to reduce oxygen to water, or to chemically combine oxygen with hydrogen atoms. It uses the energy generated by reducing oxygen to water to produce a difference in proton concentration. This concentration gradient is then used to produce ATP, but this is the work of ATP synthase, not cytochrome oxidase.

The formation of water from oxygen and hydrogen atoms involves a roundabout process as well. Rather than directly, chemically combining protons and oxygen, hydrogen atoms are separated into protons and electrons. Instead of protons being attached at once, four electrons are added to reduce oxygen to $2O_2^-$, after which protons are transferred to produce water. When this occurs, energy is released, and the active transport of protons, or proton pumping, can occur.

Let us look at this process from the aspect of cytochrome oxidase. Four electrons are necessary in the process I just mentioned, and cytochrome oxidase has two iron atoms and two copper atoms. Each of these metals can accept, release, transfer, or take one electron. Because cytochrome oxidase has four such sites, it can receive four electrons. Its state prior to receiving the electrons is called the oxidized form, and that after receiving the electrons is called the reduced form. In this way, cytochrome oxidase first accepts four electrons, and then oxygen and protons, although the order in which these are received cannot be easily explained. The active transport of protons is carried out during this process. Although this process has been known for over thirty years now, it has remained a mystery how, specifically, the electrons, oxygen, and protons are taken up.

Generally speaking, although not strictly with cytochrome oxidase, elucidating the mechanism of the enzymatic reaction in effect involves elucidating how its chemical structure, including its three-dimensional structure, changes over time and with the progression of a catalytic reaction. Let me explain how the chemical structure of enzymes, including their three-dimensional structure, is studied.

First of all, the enzyme must be isolated from the cell. Because physical and chemical methods cannot be applied unless the enzyme has been isolated, it must first simply be removed from the cell. Then, its composition and chemical structure are determined. With cytochrome oxidase as well, the first, most important step is to isolate it from the cell. Cytochrome oxidase, that is, the respiratory enzyme, was first discovered by the German scientist Warburg, who was awarded the Nobel Prize in the early 1930s. At that time, Warburg remarked that the respiratory enzyme was found inside the cell and that isolating it would be as difficult as bringing back a stone from the moon.

But here in Japan, aware of what this Nobel Prize winner was saying in Germany, Dr. Yakushiji and my teacher, Dr. Okunuki, decided to isolate cytochrome oxidase. Later, amazingly enough, they achieved isolation of the enzyme. Being the unworthy pupil that I am, I only recently obtained the paper in which Dr. Okunuki first reported isolating cytochrome oxidase.1

Figure 1 shows the method we use today to obtain cytochrome oxidase. Let me point out that, although various improvements have been made, the procedure is essentially the same as that published by Dr. Okunuki in 1941. Most importantly, the use of sodium cholate as a solubilizing agent has remained fundamentally unchanged.

In this paper, which I first looked at only after Dr. Okunuki passed away, he mentions sodium cholate. The paper was written in German, so I could not understand it all, but I was astonished to find mention of this compound. Unmistakably, Dr. Okunuki used sodium cholate in 1941. At that time, he unfortunately was not yet able to obtain cytochrome oxidase activity. Subsequently, this method was improved by Dr. Yonetani and Dr. Takemori, who are here today, and we also made a few improvements. The method of obtaining
Fig. 1 Outline of isolation procedure of cytochrome c oxidase from bovine heart muscle.

cytochrome oxidase was thus established.\(^2\)

Next, what methods of analysis should be used to study the chemical structure of cytochrome oxidase? With cytochrome oxidase, the two methods that should be used, in my opinion, are X-ray crystallography and vibrational spectroscopy, resonance Raman, or infrared spectroscopy. There are many other ways of studying cytochrome oxidase, but these are, in my view, the two most important. First, I wish to explain how X-ray crystallography is carried out. Crystals of the protein to be examined must be made. Crystals are irradiated with X-rays, producing diffraction phenomena. Since I am not a specialist in crystallography, I do not understand the subsequent steps, but some complex, mysterious calculations are made to obtain what is called the electron density. Then, further calculations are made using various models. Through this process, the structure of the crystal can be analyzed.

With this method, however, isolating crystals of the compound is an absolute necessity, and they must be crystals of good quality and of a certain size. Making crystals of a protein, however, is considered to be extremely difficult, and no method can guarantee success. For example, with cytochrome oxidase, there are many factors that affect crystallization. These factors vary greatly in importance from protein to protein. Therefore, the crystallization of a protein is difficult because we cannot predict when good crystals will be formed. We may be able to obtain good crystals tomorrow, or we may not have anything even ten years from now. For this reason, few scientists are seriously attempting the crystallization of proteins. The rate-determining step in X-ray crystallography is therefore crystallization of the protein.

In 1961, Dr. Yonetani, who is seated right over there, published a report on cytochrome oxidase crystals.\(^3\)

Dr. Tsukihara and I saw this photograph, thought we should be able to isolate the cytochrome oxidase crystal, and began our project. It provided us with the impetus we needed.

Bovine cytochrome oxidase has as many as thirteen protein subunits of different types. Cytochrome oxidase has two iron atoms and three copper atoms. It has many other components of various kinds, but, in any case, it is an enormous protein of molecular weight 210,000. Because it is so enormous, has a complex structure, and is a membrane protein—something which I will not talk about today—it takes a great deal of time to crystallize.

Although we were able to obtain crystals quite some time ago, we have had to continue trying to improve their quality. Starting from 15 Å, we were most recently able to reach 1.74 Å. This has taken us about twenty years. I wish to say, especially to the young people here today, that this represents a worst-case scenario for protein crystallization. It took us so long because we did not have such things as different types of detergents; it definitely would not take as long today.

Then, one day, we obtained these wonderful crystals of a truly beautiful color. This shows X-ray diffraction at up to 2.6 Å resolution. I was astounded that, in less than ten months after we had obtained this crystal, Dr. Tsukihara, whom I mentioned earlier, was able to show us its structure (Fig. 2).\(^4\) Figure 2 shows only the main chain of the peptide. In the actual crystal, this enzyme is in a dimer. Each monomer has a molecular weight of 210,000, and there are 13 different types of protein subunits, which are indicated in different colors as shown in Fig. 2. The portion composed mainly of \(\alpha\)-helices is thought to be embedded in the membrane (Fig. 2A). A top view of this structure (Fig. 2B) shows a fairly large space between the two monomers.

Figure 3 shows the location of the metals involved in the oxidation-reduction mechanism to which we paid particular attention: heme \(a\), and heme \(a_3\), which are iron, and Cu\(B\) and Cu\(A\). The three-dimensional structures of these metal sites became clear as well.\(^5\)

Cytochrome oxidase reaction is involved in the reduction of oxygen to water and in the proton pump, or the active transport of protons from the inner side of the mitochondrial inner membrane to the outer side. I will now explain how the X-ray structure of cytochrome oxidase, contributes first to the oxygen reduction mechanism and then to the active transport of protons.

Figure 4 indicates the unique chemical properties of the oxygen molecule. Oxygen does not readily undergo one-electron reduction, so such a process would be extremely slow. An energy source, of course, would make a difference, but usually the reaction is highly unlikely to occur. Two-electron reduction, however, which involves the reduction of oxygen with two electrons at once, producing \(O_2^{2-}\), is known to be an extremely rapid
Hypotheses about where oxygen binds to cytochrome oxidase were already being advanced thirty-some years ago, long before the X-ray structure of cytochrome oxidase became known. At that time, it was known that the oxygen formed a bond with iron and that a copper ion existed very near this oxygen-binding site. It was Professor Winslow Caughey, whom I introduced earlier, who first recognized the importance of the proximity of this copper ion to the oxygen-binding site. That is, the location of this copper ion makes two-electron reduction possible. Two electrons can be transferred simultaneously, one from the iron and another from the copper, to produce a γ-peroxo intermediate, as shown in Fig. 5. Specifically, Dr. Caughey noted the importance of the proximity of CuB and nearly thirty years ago proposed the bridging peroxide mechanism, stating that oxygen is reduced by way of a γ-peroxo intermediate.

Because this bridging peroxide mechanism is extremely clear, it was readily accepted by many people working in the field of cytochrome oxidase. Although most people recognized the formation of the γ-peroxo intermediate, they failed to notice an important point. That is, assuming that this mechanism is correct, the reaction that would produce the γ-peroxo intermediate should be extremely rapid. The distance between the oxygen and copper is approximately 2 Å. The rate of
the reaction would be limited by the distance over which the electron is transferred, and, with the electron moving over a distance of only 2 Å, the reaction can be estimated to take only about a pico second, if all goes well. In other words, it should be almost impossible to detect this oxygen-bound form experimentally.

However, astonishingly enough, the oxygen-bound form was discovered by resonance Raman studies in collaboration with Dr. Kitagawa and Dr. Ogura. I had never been so surprised in my life as when I learned that they had detected this form. Furthermore, they showed that the half-life of the form is 0.4 msec. While we had thought that the speed would be about a pico second, it was in fact about 0.4 msec. Although this is still rather fast, it is extremely slow if we assume that the bridging peroxide mechanism is correct. Why is this oxygen-bound form so stable? The crystal structure gives us a very important indication of the answer.

Figure 6 shows the coordination structure of Cuβ, the copper atom close to the oxygen-binding site. This is the reduced state, in which the Cuβ is in a state to which the oxygen will bind. In this state, only three histidine ligands are present at the site, and, moreover, they are in trigonal, or triangle planar form. According to inorganic chemistry, a Cu1+ complex with a triangle planar structure is extremely stable. In the stable state, it is difficult to transfer electrons there or to accept a new ligand. We think that this is the reason for the abnormal stability of the oxygen-bound form.

Looking again at the X-ray crystal structure, we found that the tyrosine near the oxygen binding site is bound by a covalent bond to one of the histidine ligands of Cuβ. As a result, the OH group of the tyrosine is in close proximity to the oxygen-binding site, as shown in Fig. 7. Using a model, we see that oxygen can bind in a way that hydrogen bonds can form quite adequately. Thus, although we had thought for over thirty years that when oxygen binds to cytochrome oxidase it forms a bridge between iron and copper, these X-ray structures showed that the bridge is in fact formed between the iron and the tyrosine. Electrons are unlikely to be transferred from Cuβ to the bound oxygen. Whereas Cuβ is thought to become involved at a subsequent stage, there does not seem to be any electron transfer at least in this initial stage. So, the crystal structure strongly indicates that, at least in the initial stage of the mechanism of oxygen reduction by cytochrome oxidase, a hydrogen bond is formed with the OH group of tyrosine. Moreover, the electron in fact does not come from Cuβ but from another iron near this iron to form a hydroperoxo form, Fe^{3+}O-O-H. I believe such an in-
termediate form was entirely unexpected until the X-ray structure became apparent.

Next, I wish to examine the proton pump, or the active transport of the proton. One of the most important aspects in understanding the mechanism of the proton pump, is identifying the changes that occur in X-ray structure coupled with the oxidation-reduction reaction.

Figure 8 shows the redox coupled conformational change of the enzyme detectable in the X-ray structure.\textsuperscript{8} The oxidized state is shown in red, and the reduced state in green. Dots indicate the surface of the molecule on the outer surface of the inner mitochondrial membrane in the oxidized state. While many changes occur, we feel the most important is that which takes place in the amino acid called aspartic acid-51.

In the oxidized state, aspartic acid-51 is buried completely inside the molecule, so that it does not come into contact at all with the water in the outer part of the mitochondrion. When reduction occurs, however, the COOH group of the aspartic acid moves to the surface of the molecule and comes into contact with the water of the outer part of the mitochondrion. So, although this group is at first not accessible, it becomes accessible during the reduction phase of the oxidation-reduction reaction. Furthermore, in the oxidized state, the carboxyl group of aspartic acid-51 is bound by a hydrogen bond network to the inner side of the inner mitochondrial membrane as shown in Fig. 9.\textsuperscript{8}

A hydrogen bond inside a protein is considered an excellent pathway for protons. Thus, because it is connected by a network of hydrogen bonds, aspartic acid-51 is able to take up protons from the inner side of the inner mitochondrial membrane and in that sense is accessible to the inner part of the mitochondrion. In the reduced state, aspartic acid-51 is accessible on the outer side. When this happens, however, the hydrogen bond connecting with the hydrogen bond network is broken, so it is accessible only on the outer side. In this way, the accessibility of aspartic acid-51 changes during the course of the oxidation-reduction reaction.

Even more importantly, the protonated carboxyl group, or COOH, at the end of the aspartic acid molecule is found to a considerable degree as COO\textsuperscript{-}, that is, in a dissociated state, in aqueous solution. In protein, however, in places where the dielectric constant is low, it is almost always found as COOH, not COO\textsuperscript{-}, showing high affinity for the proton. In the oxidized state of cytochrome oxidase, the COO\textsuperscript{-} is in the inside of the protein, so it shows high affinity for protons. In the reduced state, however, it becomes exposed on the surface of the molecule, and, since this is almost the same as being in aqueous solution, the affinity is reduced.

Table 1 summarizes the actions of the carboxyl group of aspartic acid-51. In the oxidized state, it is accessible to the interior of the mitochondrion in the sense that it can take up protons from the inner part. In that state, it is located inside the protein, so its affinity must be extremely high. In the reduced state, however, it is accessible only to the outer part of the mitochondrion, and because it is surrounded by water, its affinity...
Table 1  Summary of the Redox-coupled Conformational Change in Carboxyl Group of Asp51 in Subunit I of Bovine Heart Cytochrome c Oxidase

<table>
<thead>
<tr>
<th></th>
<th>H-bond</th>
<th>Accessibility</th>
<th>pK</th>
</tr>
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<tbody>
<tr>
<td>fully oxidized state</td>
<td>4</td>
<td>matrix side</td>
<td>high</td>
</tr>
<tr>
<td>fully reduced state</td>
<td>1</td>
<td>intermembrane side</td>
<td>low</td>
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for the proton is reduced.

Thus, the crystal structure strongly suggests that the carboxyl group of aspartic acid-51 is the site of the proton pump that takes up the proton from the interior of the mitochondrion in the oxidized state and releases it in the exterior part of the mitochondrion in the reduced state. Needless to say, however, this crystal structure has been determined only in static, oxidized, and reduced states, not during enzyme turnover. Therefore, although these studies strongly indicate that the carboxyl group of aspartic acid-51 is the site of the proton pump, much more research is needed to prove that the proton really does pass through this site and that this site acts as the proton pump. To do so, we will need to use not only X-ray crystallography but also different spectroscopic methods, as well as currently exploiting techniques of altering the aspartic acid-51 amino acid by mutation. In this way, we should be able not only to determine the correctness of this theory but also make many new discoveries regarding the mechanism of the cytochrome oxidase reaction.

References