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