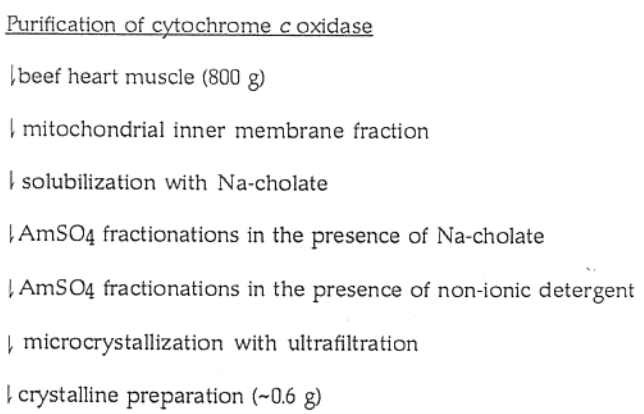

Fig. 1 Outline of isolation procedure of cytochrome *c* oxidase from bovine heart muscle.

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 sometime 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 α-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