8. Absorption Spectra of Cytochrome b_5 in Crystalline and Dissolved States

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Cytochrome b_5 is a hemoprotein which exists in various organs of animals. Although the physiological role of this cytochrome is not yet known, its content in mammalian liver is remarkably high, even more than the well known cytochrome a , b , c , c_1 etc. In 1959, Raw and Colli reported crystallization of pig liver cytochrome b_5 in a short communication¹⁾ but the full paper of this work has not yet been published. We recently established a method for the crystallization of cytochrome b_5 from rabbit livers.^{2),3)} The method is reproducible and thus provides the way to study the properties of cytochrome b_5 molecule in crystalline state as well as in dissolved state.

Since the stereo structure of a protein molecule is determined only in crystalline state by X-ray analysis, it is important to examine the similarity of the molecular structure in crystal and solution by other physical methods. In the case of hemoproteins, one method is to compare the spectra. From various spectrophotometric studies it is generally assumed that the electronic structure of the heme prosthetic group is essentially identical in crystal and in solution. However, significant spectroscopic differences between crystalline and dissolved yeast cytochrome c peroxidase at ferric state was recently reported by Yonetani.⁴⁾ In the present investigation a similar result was also obtained with cytochrome b_5 in ferro state but not in ferric state.

Fig. 1. Crystalline ferricytochrome b_5 obtained from the first band on the DEAE-cellulose column in the trypsin extraction method (see text).

Methods. The crystalline preparation (Fig. 1) of rabbit-liver cytochrome b_5 used in this study was the one obtained from the faster moving band in the chromatography on DEAE-cellulose column of trypsin extraction method.^{2),3)} The absorption spectra of the dissolved cytochrome b_5 at room temperature was measured by a sensitive split-beam spectrophotometer.^{5),6)} Those at liquid nitrogen temperature (-196°C) were measured after dilution of the cytochrome solution with an equal volume of glycerol according to the method of Estabrook.⁷⁾

The spectra of the cytochrome in crystalline state at room temperature were measured in the following three ways: 1. In the case of oxidized form, the sample cuvette (2 mm path) contained crystalline ferricytochrome b_5 suspended in saturated ammonium sulfate solution containing phosphate buffer at pH 7.4, and the reference cuvette contained the saturated solution of ammonium sulfate. The spectrum was recorded by the split-beam spectrophotometer. For measuring the spectrum of reduced cytochrome b_5 , a trace of sodium dithionite was added to the suspension of the crystals. 2. Suspension of the crystals in the saturated ammonium sulfate solution was put in

Fig. 2. Absorption spectra of reduced cytochrome b_5 in crystalline and dissolved states at room temperature. Concentration of cytochrome b_5 was adjusted in such a way that the $E_{556\text{m}\mu}-E_{600\text{m}\mu}$ in solution and $E_{559\text{m}\mu}-E_{600\text{m}\mu}$ in crystals were nearly equal (E , extinction).

between two glass slide and the spectra were measured similarly as the method 1. 3. Spectra of a single crystal was recorded by Zeiss Ultramicrospectrophotometer.

The spectra of cytochrome in the crystalline state at low temperature (-196°C) was measured by the split-beam spectrophotometer after cooling the cuvette containing the suspension of the crystals with liquid nitrogen.

Results. The visible absorption spectra of solution and crystalline suspension of ferrocytochrome b_5 are shown in Fig. 2. Two remarkable differences, height of the Soret band and shape of the α -bands, were observed in the spectra of the two states. It is assumed that the first difference is induced simply by the "flattening effect" due to the ununiform distribution of the pigment in the suspension of crystals. Careful inspection of the two spectra shows that the α band in crystals is also lowered but β band is not reduced proportionally (Fig. 3). Similar result was also obtained with the suspension of crystals placed in between two glass plates (Method 2), but in the case of the spectrum of a single crystal (Method 3), the Soret band was not lowered so remarkably as found in the above two cases. These results support the above assumption.

Difference in shape of the spectra of ferrocytochrome b_5 at the

Fig. 3. Absorption spectra in α and β region of reduced and oxidized cytochrome b_5 in solution and crystals. Equal concentration (approx. $2.2 \mu\text{M}$) of cytochrome b_5 was used in all cases.

α -region between crystalline and dissolved states is important, since this suggests the presence of some different electronic structure in these two states. The peculiar shape of the α -band with a shoulder (561 $m\mu$) near the peak (556 $m\mu$) of ferrocytochrome b_5 solution is a remarkable feature as compared to other cytochromes. However, in the spectrum of suspension of crystals, the shape of the α -band is nearly symmetrical (peak at 559 $m\mu$) like the other cytochromes. It seems that the electronic structure, responsible for the 556 $m\mu$ peak in dissolved ferrocytochrome b_5 , mostly disappeared in crystals. Essentially the same shape of α -band was observed with the crystals placed between two glass plates (Method 2) and also with a single crystal (Method 3).

As shown in Fig. 3, no essential difference was observed in the spectra of ferricytochrome b_5 in crystalline and dissolved states at room temperature.

When the spectrum of dissolved ferricytochrome b_5 was measured at liquid-nitrogen temperature (-196°C) no remarkable difference was observed in comparison to the spectrum at room temperature,

Fig. 4. Low temperature spectra (-196°C) of cytochrome b_5 . Concentrations of cytochrome b_5 were arbitrary.

although all shoulders and peaks became more distinct (Fig. 4, C). Very similar spectrum was obtained with ferricytochrome b_5 in crystals. In the case of ferrocytochrome b_5 in dissolved state, the peak and shoulder (556 $m\mu$ and 561 $m\mu$ respectively) observed at room temperature, shifted toward shorter wave-length by about 4 $m\mu$ and were separated distinctly into two peaks at -196°C (Fig. 4, B). Essentially similar spectrum was observed even with the suspension of the crystals (Fig. 4, A).

The above results together with those at room-temperature suggest the presence of some difference in electronic structure between crystalline and dissolved state of ferrocytochrome b_5 molecule at room temperature, but such difference diminished almost completely at liquid nitrogen temperature.

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