Vol. 97, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 17, 1980 Pages 22-27

A REVERSIBLE SPIN CONVERSION OF CYTOCHROME b_5 AT HIGH TEMPERATURES

Toshihiro Sugiyama, Retsu Miura, Toshio Yamano,
Kiyoshi Shiga*, and Hiroshi Watari*

Department of Biochemistry, Osaka University Medical School
Nakanoshima 4-3-57, Kita-ku, Osaka, Osaka 530, Japan
and
*National Institute for Physiological Sciences
Myodaiji, Okazaki, Aichi 444, Japan

Received September 15,1980

SUMMARY: The thermodynamics for the reversible spin conversion of cytochrome b_5 have been studied by absorption and circular dichroic spectroscopy in the temperature range from 15 to as high as 83°C. The optical absorption spectrum of the oxidized cytochrome b_5 below 45°C, predominantly of low-spin type, changes to an essentially high-spin type on increasing the temperature. This spectral transition of cytochrome b_5 was found to take place in a relatively limited range from 45°C to 65°C. The circular dichroic spectrum of cytochrome b_5 at high temperatures is that of partially unfolded polypeptide with an exposed heme moiety. All these spectral changes were reversible.

Cytochrome b_5 is well known to be the intermediate electron carrier in the microsomal NADH-dependent stearyl coenzyme A desaturase system (1-3). Recently, more extensive studies on the participation of cytochrome b_5 in the hepatic mixed function oxidase system have been carried out, and revealed that cytochrome b_5 plays a key role in certain drug hydroxylation systems (4,5). Consequently, knowledge of the structure and function of cytochrome b_5 is required for understanding the mechanism of electron transport in the microsomes.

The heme iron in both ferrous and ferric cytochrome b_5 is in its low-spin state (6). However, from spectral and EPR studies cytochrome b_5 reversibly changes to a high-spin state below pH 4 at temperatures between 20 and 296 K, presumably because of heme dissociation (7). Above pH 12 it also changes reversibly to another type of low-spin state as a result of dislocation of the heme (7). The heme environment of cytochrome b_5 was also studied by chemical modification (8), X-ray crystallography (9), and NMR (10, 11).

0006-291X/80/210022-06$01.00/0

The optical absorption spectrum of cytochrome b_5 is known to change with variation in pH (6,7) and temperature (7, 12, 13). The correlation between the optical spectra and the spin states of cytochrome b_5 has not yet been established. So, it is of interest to see whether the absorption spectrum of cytochrome b_5 changes with increasing temperature above room temperature. The effect of thermal treatment on the environment of heme crevice and on the secondary and tertiary structures of cytochrome b_5 has now been examined by the absoption and circular dichroic measurements.

MATERIALS AND METHODS: Detergent solubilized cytochrome b_5 was purified from rabbit liver microsomes according to the methods of Spatz and Strittmatter (14), with some modifications (5). The purified cytochrome b_5 contained 55 nmoles of heme per mg protein. The concentration of cytochrome b_5 was determined from the intensity of Soret band at 413 nm of the oxidized form using a millimolar extinction coefficient of 117 cm^{-1} mM^{-1} (15). Absorption measurements were performed with a Varian-Cary 119 spectrophotometer, equipped with a thermojunction. The temperature was continuously raised from approximately 15 to 83°C over a period of 60 min by using a water-jacketed cell holder and a water bath. The temperature in the cuvette was monitored with a thermistor. The circular dichroism (CD) spectra were recorded with a JASCO J-40 A circular dichroic spectrophotometer using quartz cells of 1.0 mm light path below 300 nm and 10 mm light path above 300 nm. A water-jacketed cell holder was employed during the investigations of the effect of temperature variation.

RESULTS: Fig. 1 shows the absorption spectra of oxidized cytochrome b_5 in the region from 240 to 700 nm at various temperatures. Spectra below 45°C, having a β-band at 530 nm and an α-band at 560 nm, are typical of the low-spin type of ferric hemoprotein (16). Above 45°C, the spectrum of the low-spin type decreased, and new peaks appeared at 637 nm and 510 nm. Since these peaks are characteristic of ferric high-spin (16), oxidized cytochrome b_5 seems to undergo a transition from low-spin to high-spin states on increasing the temperature. The absorption spectrum in the Soret region for cytochrome b_5 below 45°C is that of the native cytochrome b_5, while above 45°C the Soret absorptivity is reduced with a blue shift by 25 nm. This spectral transformation of oxidized cytochrome b_5 is characterized by the isosbestic points at 394, 508, and 582 nm. These transitions were found to be reversible at the temperatures below 65 °C. Fig. 2 illustrates the temperature dependence of the absorbance changes

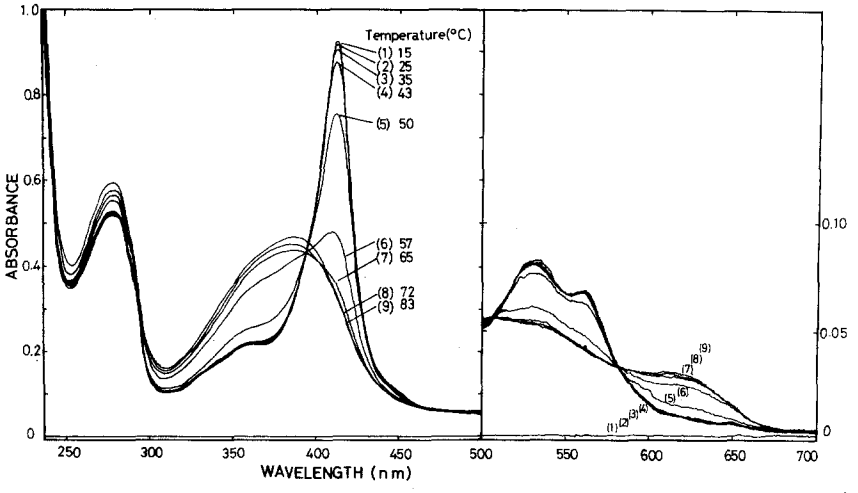

Fig. 1. Optical absorption spectra of cytochrome b_5 at temperatures from 15°C to 83°C. The concentration of cytochrome b_5 was 7.8 μM in 0.1 M potassium phosphate, pH 7.4 . Temperatures(°C) were:(1)15, (2)25, (3)35, (4)43, (5)50, (6)57, (7)72, (8)83. At each temperature, preincubation was carried out for 5 min to establish the equilibrium, and then absorption spectrum was recorded.

with maximum absorbances taken as 100 per cent at 388, 413, 533, 620 nm, where the midpoint of this transition is observed at 55°C independent of its wavelength. Above 65°C, the spectral changes are accompanied by a slow irreversible process resulting in the loss of the isosbestic points.

Figs. 3-A and 3-B show CD spectra of oxidized cytochrome b_5 in the Soret region and the ultraviolet region, respectively. Fig. 3-A, curve 1 has a

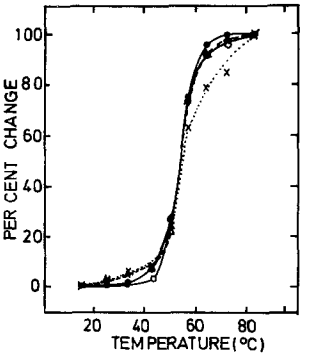

Fig. 2. The temperature dependence of the absorbance change of cytochrome b_5. The data of Fig. 1 are replotted against temperature in per cent of the maximal changes at the following wavelengths (nm):(X)388, (●)413, (Δ)533, (○)620.

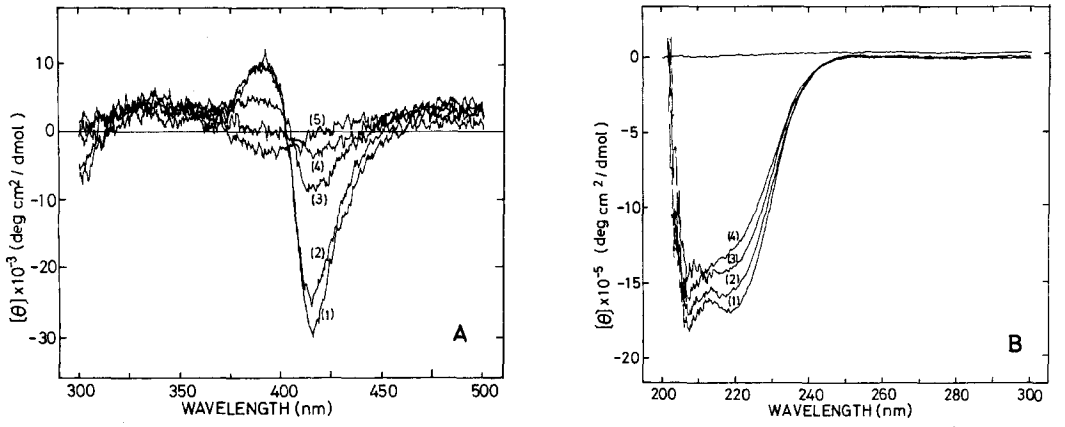

Fig. 3. Circular dichroic spectra of cytochrome b_5 at temperatures from 25°C to 83°C. A: Visible CD spectra. The concentration of cytochrome b_5 was 6.6 μM in 0.1 M potassium phosphate, pH 7.4, and light path was 10 mm. Temperatures (°C) were:(1)25, (2)40, (3)56, (4)60, (5)70. B: Ultraviolet CD spctra. The concentration of cytochrome b_5 was 10 μM in 0.1 M potassium phosphate, pH 7.4, and light path was 1.0 mm. Temperatures (°C) were:(1)25, (2)55, (3)65, (4)83.

negative extremum with strong intensity at 415 nm and a positive maximum with moderate intensity at 390 nm which are characteristic of low spin type of cytochrome b_5 (17-19). When the temperature was raised from 25°C to 70°C, the CD spectrum transformed to curve 5 which shows a negative CD with small intensity at 390 nm and no ellipticity at 415 nm. The plot of the negative CD intensity at 415 nm (data not shown) agaist temperature, as in Fig. 2, manifested the transition temperature at 55°C which is in agreement with that observed in Fig. 2.

On the other hand, the far-ultraviolet CD bands were monotonically decreased with raising the temperature (Fig. 3-B). Even after 10 min-incubation at 83°C, the ellipticity at 218 nm remained 80% of the original one. It is noteworthy that these CD changes resemble those observed in the reversible denaturation experiments with guanidine hydrochloride (17,18), or with sodium dodecyl sulfate (17), indicating these CD changes may be due to the partial unfolding of the protein. Futhermore, the CD changes in these regions were less conspicuous than in the Soret region and were linear with the temperature change without transitional characteristics.

DISCUSSION: Oxidized cytochrome b_5 around 55°C was found to be in the low-spin and high-spin states, indicating the presence of thermal mixing of the low-spin and high-spin states. Since the absorption spectra of cytochrome b_5 observed at high temperatures were found to be very similar to those of cytochrome b_5 at low pH (7) or at high pH (6), the high-spin state observed above the transition temperature is considered to be due to the dislocation of heme in the cytochrome. Remarkable changes of the CD spectrum in Soret region and small changes in far-ultraviolet region resemble those observed in the reversible denaturation process of cytochrome b_5 with various denaturants (17-19). It can be assumed that this process represents a change in the environment of the heme without significant alteration in the structure of protein moiety of the cytochrome.

The transition between the high- and low-spin types was found to be reversible under the conditions employed. The conversion rate of low- to high-spin state was very rapid under these conditions (within a few minutes), while the reverse reaction was rather slow and took at least 10 min-incubation for completion.

REFERENCES:

1. Oshino, N., Imai, Y., and Sato, R. (1966) Biochim. Biophys. Acta 128, 13-28
2. Shimakata, T., Mihara, K., and Sato, R. (1972) J. Biochem. 72, 1163-1174
3. Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B., and Redline, R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4565-4569
4. Imai, Y. (1979) J. Biochem. 86, 1697-1707
5. Sugiyama, T., Miki, N., and Yamano, T. (1980) J. Biochem. 87, 1457-1467
6. Bois-Poltoratsky, R., and Ehrenberg, A. (1967) Eur. J. Biochem. 2, 361-365
7. Ikeda, M., Iizuka, T., Takao, H., and Hagihara, B. (1974) Biochim. Biophys. Acta 336, 15-24
8. Strittmatter, P. (1960) J. Biol. Chem. 235, 2492-2497
9. Mathews, F. S., Levine, M., and Argos, P. (1972) J. Mol. Biol. 64, 449-464
10. Keller, R. M., and Wüthrich, K. (1972) Biochim. Biophys. Acta 285, 326-336
11. Keller, R. M., and Wüthrich, K. (1980) Biochim. Biophys. Acta 621, 204-217
12. Hagihara, B., and Iizuka, T. (1971) J. Biochem. 69, 355-362
13. Kajihara, T., and Hagihara, B. (1968) Structure and Function of Cytochromes pp. 581-593, Okunuki, K., Kamen, M. O., and Sekuzu, I. (eds.) Univ. Tokyo Press, Tokyo
14. Spatz, L., and Strittmatter, P. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1042-1046
15. Ozols, J., and Strittmatter, P. (1964) J. Biol. Chem. 239, 1018-1023
16. Iizuka, T., and Yonetani, T. (1970) Advan. in Biophys. 1, 157-182

17. Schnellbacher, E., and Lumper, L. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 615-628
18. Huntley, T. E., and Strittmatter, P. (1972) J. Biol. Chem. 247, 4641-4647
19. Tajima, S., Enomoto, K., and Sato, R. (1976) Arch. Biochem. Biophys. 172, 90-97