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Absorption Spectra of Highly Purified Liver Microsomal Cytochrome P-450 in Non-Equilibrium Conformational States at Low Temperatures

S. Greschner

Central Institute of Molecular Biology, Academy of Sciences of the GDR, DDR-1115 Berlin-Buch, German Democratic Republic

Abstract. Absorption spectra of highly purified liver microsomal cytochrome P-450 in non-equilibrium states were obtained at 77 K by reduction with trapped electrons, formed by gamma-irradiation of the water-glycerol matrix. In contrast to the equilibrium form of ferrous cytochrome P-450 with the heme iron in the high-spin state the non-equilibrium ferrous state has a low-spin heme iron. The absorption spectrum of the non-equilibrium ferrous cytochrome P-450 is characterized by two bands at 564 (α-band) and 530 nm (β-band). When the temperature is increased to about 278 K this non-equilibrium form of the reduced enzyme is relaxed to the corresponding equilibrium form with a single absorption band at 548 nm in the visible region characteristic for a high-spin heme iron.

Key words: Cytochrome P-450 – Absorption spectra – Reduction – Non-equilibrium conformational states

1. Introduction

The term "cytochrome P-450" refers to a group of heme-containing proteins catalyzing the hydroxylation of various structurally different compounds (Ishimura 1978). The essential catalytic function of the multi-step reaction cycle is based on the capability of the enzyme system to activate molecular oxygen. This activation is achieved by inserting two electrons into the oxygen molecule (Ishimura 1978).

Recently the concept was proposed that conformational non-equilibrium states could play an important role in the catalytic function of enzymes (Blumenfeld and Davidov 1979). The aim of the present study is to prove if such non-equilibrium states can appear also in cytochrome P-450. Therefore, the influence of the oxidation state of the heme iron of cytochrome P-450 on the ligand environment of the protein was studied by means of the absorption spectroscopy at liquid nitrogen temperatures (Blumenfeld et al. 1974;

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Blumenfeld and Davidov 1979; Greschner et al. 1979). Under conditions of limited mobility at 77 K the ferric heme iron of cytochrome P-450 was reduced by trapped electrons arising during gamma-irradiation of protein solutions in water-glycerol mixtures. Under these conditions kinetically stabilized non-equilibrium states are found, in which the iron of the active centre is reduced and the protein remains in the "oxidized" conformation. The differences in the spectral characteristics of the chemically and physically (gamma-irradiation at liquid nitrogen temperature) reduced proteins indicate that not only the redox state of the heme iron but also its environment was changed during the redox reaction.

We report here data on highly purified microsomal cytochrome P-450 LM_2 from rabbit liver without contaminating proteins cytochromes b_5 and P-450 isozymes content which are in agreement with previous data indicating significant changes of the heme iron environment during redox reactions in partially purified cytochrome P-450 including the involvement of the protein moiety in this process (Greschner et al. 1979).

2. Materials and Methods

Cytochrome P-450 was isolated from rabbit liver microsomes with slight modifications (Karuzina et al. 1979) according to the method of Imai and Sato (1974). The isolated cytochrome P-450 contained 18 nM hemoprotein per mg protein and was free of both cytochromes b_5 and P-420 (Karuzina et al. 1979). Concerning the spectral properties this cytochrome P-450 is similar to the LM_2-form, prepared by the Coon group (Haugen and Coon 1976; Karuzina et al. 1979).

The absorption spectra were measured at 77 K in cylindrical quartz vessels of diameter 5 mm, placed in a special quartz dewar with liquid nitrogen. We used an adapted "Specord UV-VIS" spectrophotometer (GDR). In order to obtain a matrix optically transparent at 77 K, a mixture of glycerol and phosphate buffer (0.1 M, pH 7.0) 3 : 2 on weight basis was used. The cytochrome P-450 concentration amounted to 2×10^{-5} M. At room temperature the protein was reduced with sodium dithionite (Merck, FRG). The low temperature reduction of the aqueous glycerol solution of cytochrome P-450 was carried out at 77 K with trapped electrons formed by Co-60-gamma-irradiation of the matrix. The dose rate was 38 kGy/h. The absorption of trapped electrons in the samples was removed by bleaching with visible light from a tungsten lamp. The irradiation dose was varied from 10 to 100 kGy. At doses of about 60 kGy the degree of enzyme reduction reached the saturation level. The irradiation with such doses at 77 K does not affect the spectral characteristics of the dithionite reduced cytochrome P-450. Therefore, possible radiation damages do not influence the optical properties of the protein.

The solutions were de-aerated before gamma-irradiation by the freezing-thawing procedure (3 cycles, applying a vacuum of about 10^{-5} mm Hg to the frozen state, at 77 K). In special experiments was shown that the spectra at 77 K were not influenced by the presence or absence (de-aerated sample) of

oxygen. An effect of oxygen was found on heating the samples up to room temperature (see "Results and Discussion"). For further methodical details see (Blumenfeld et al. 1974; Greschner et al. 1979). All chemicals used were of analytical grade.

3. Results and Discussion

In Fig. 1 the absorption spectrum of the oxidized cytochrome P-450 (curve A) is compared with the reduced spectra obtained by dithionite (reduced at room temperature, measured at 77 K; curve B) and by gamma irradiation at 77 K (curve C), respectively. The absorption spectrum of the oxidized (ferric) form of the protein (Fig. 1, curve A) is characterized by two maxima at 534 and 570 nm. These bands are caused by the low-spin component of the oxidized cytochrome P-450 (Haugen and Coon 1976). Taken together with the very weak absorption in the 645 nm region, it can be assumed that at 77 K under our conditions the heme iron of the oxidized enzyme is mainly in the low-spin state (Haugen and Coon 1976).

The absorption spectrum of the chemically (by dithionite) reduced form (referred as "equilibrium form" in the following) in this spectral region consists of one broad band with a maximum at 548 nm (Fig. 1, curve B). The gamma-irradiation of the water-glycerol solution of the dithionite-reduced cytochrome P-450 at 77 K has no effect on the spectral characteristics of the protein in the investigated spectral range (500–700 nm).

As a result of gamma-irradiation of ferricytochrome P-450 at 77 K the reduction of the protein is observed (Blumenfeld et al. 1974; Greschner et al. 1979). However, the absorption spectrum of the hemoprotein reduced at 77 K (Fig. 1, curve C) differs strikingly from the corresponding spectrum of the reduced equilibrium form of the protein (Fig. 1, curve B) and is characterized by two maxima at about 530 and 564 nm.

The occurrence of maxima at about 530 and 564 nm in the absorption spectrum of the low temperature reduced cytochrome P-450 could be explained by the difference in the structure of the environment of the heme iron in the oxidized and reduced form of the protein. Due to the limited mobility in the macromolecule at 77 K the ligand environment of the heme iron in the cryogenic temperature reduced protein differs hardly from that in the oxidized form (Blumenfeld and Davidov 1979). The absence of an influence of gamma-irradiation on the spectral properties of the dithionite reduced form of cytochrome P-450 excludes, as an alternative explanation, the occurrence of maxima at 530 and 564 nm by radiation damages in the enzyme. Heating of the sample reduced by gamma-irradiation at 77 K results in the disappearance of the maxima at 530 and 564 nm and leads to the formation of the typical ferrous cytochrome P-450 equilibrium spectrum as a transient form at about 278 K (very similar to Fig. 1, curve B). The final spectrum of cytochrome P-450 at room temperature (298 K) is represented by the oxidized form in the presence of oxygen or by the P-450-CO-complex in the absence (de-aerated sample) of oxygen. The CO is formed on gamma-irradiation of water-alcohol mixtures at 77 K (Dainton et al.

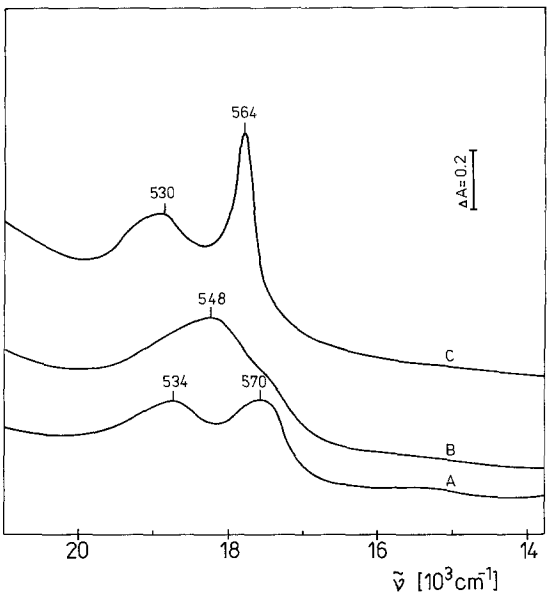

Fig. 1A–C. Absorption spectra of highly purified cytochrome P-450 at 77 K in the visible region: **A** oxidized cytochrome P-450, **B** chemically reduced by dithionite at 298 K, measured at 77 K, **C** physically reduced by gamma-irradiation at 77 K. Concentration of cytochrome P-450: 0.02 mM. The measuring unit of the numbers at the absorption peaks is nm. For other details see text

1969). The spectra at 77 K (Fig. 1) were not influenced by oxygen, as mentioned in "Materials and Methods".

From the spectral differences it can be concluded that significant changes of the heme iron environment during redox reactions in cytochrome P-450 occur. This conclusion is supported by preceding CD-measurements (Ruckpaul et al. 1977).

These data indicate the formation of a kinetically stabilized non-equilibrium form at reduction of cytochrome P-450 by gamma-irradiation at 77 K, which is structurally different from the equilibrium form of the reduced protein.

The non-equilibrium state of highly purified reduced cytochrome P-450 is characterized by a single α-band at 564 nm in the spectrum (Fig. 1, curve C). Therefore, the split α-band found in preliminary investigations with a partially purified cytochrome P-450 (Greschner et al. 1979) originates from cytochrome b_5 still present in the preparation.

In the oxidized form the heme iron of cytochrome P-450 LM_2 is attached by two axial ligands to the protein (Cramer et al. 1978), resulting in a low-spin state of the metal ion (Haugen and Coon 1976). After reduction, in analogy with the soluble bacterial cytochrome P-450 (Champion et al. 1975) the microsomal cytochrome P-450 should have its iron in the high-spin state (S = 2). The existence of bands in the visible spectrum of the non-equilibrium form of ferrous cytochrome P-450 at 530 (β-band) and 564 nm (α-band) points to the low-spin character of the heme iron in the low temperature reduced enzyme (Magonov et

al. 1978; Gasyna 1979). This indicates, as a rule, the existence of two axial ligands in the coordination sphere of the heme iron (II) (Hoard 1975; Magonov et al. 1978; Gasyna 1979). As a hemoprotein in these two spin states differs in the position of the heme iron relative to the heme plane (Hoard 1975), and assuming that this is valid also for cytochrome P-450, a conformational change in the relaxation process from the reduced low-spin (non-equilibrium form) to the reduced high-spin (equilibrium form) cytochrome P-450 is expected. The protein is involved in this process via the heme iron ligands (Cramer et al. 1978) and possibly by its porphyrin contacts (Ruckpaul et al. 1977).

The experimental data indicate a difference in the environment of the heme iron in the oxidized and the reduced form of the enzyme. This fact suggests the active role of the protein in the catalytic cycle (Blumenfeld and Davidov 1979).

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