THE REDOX POTENTIAL OF LIVER CYTOCHROME P-450*

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SUMMARY

The redox potential of phenobarbital-induced rabbit liver cytochrome P-450 has been measured, within microsomes, using dyestuff reduction under anaerobic conditions, and optical and ESR spectrophotometric techniques for determining the ratios of oxidants and reductants in the system. An apparent midpoint potential of about -0.41 volt at pH 7.0 was found. The uncertainty concerning the exact E'_o resulted from inability to achieve titration potentials by the dyestuff technique, below -0.44 volt.

Cytochrome P-450, a b-type CO binding and autoxidizable hemoprotein, has been identified as an oxygen-activating enzyme in mixed function oxidations occurring in liver and adrenal gland (1). The optical (2, 3, 4) and electron spin resonance (ESR) spectra (5) of the substance have been relatively well characterized, but the redox properties are still unknown. In this study we have attempted to determine the redox potential of the cytochrome as it occurs in membrane in order to throw light upon pathways of microsomal electron transport, and upon possible mechanisms of interaction of the reduced cytochrome with O_2.

EXPERIMENTAL METHODS

Rabbit liver microsomes were prepared by the method of Mason and his co-workers (6), after phenobarbital injections. The whole

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microsomal pellet consisting of rough and smooth microsomes was employed. These preparations contained 2.2 to 3.3 mumoles cytochrome P-450 per mg. protein.

Redox potentials were determined by the following technique: a sample of microsomes dispersed in 0.1 M phosphate buffer pH 7.0 (0.1 ml., at about 18 mg. protein per ml.) was placed in an anaerobic ESR tube (7) which had been evacuated and flushed with oxygen-free hydrogen. An aliquot of dyestuff, 0.2 ml., 3 x 10^{-3} M, which had been reduced with standard sodium dithionite solution to a pre-determined ratio of oxidized to reduced dyestuff, was then added anaerobically, and the system allowed to come to equilibrium. The concentration of reduced dyestuff was then measured at room temperature, using a Cary model 14 spectrophotometer equipped with a scattered transmission accessory and a special holder for ESR tubes. The sample was then rapidly frozen in liquid nitrogen and the ESR spectrum of the sample in the high field region (microsomal Fe_x, or oxidized cytochrome P-450) (8) was determined at -180°, using a Varian V-4500 spectrometer. The ratio of oxidized to reduced cytochrome P-450 was determined from the signal height of the g = 2.25 line of the ESR spectrum. The optical and ESR measurements were repeated at 15 minute intervals to insure the attainment of equilibrium in the redox reaction, but this was usually obtained within the first 15 minutes.

Redox potentials were calculated from the observed ratio of oxidized to reduced dyestuff (at room temperature) and of oxidized to reduced cytochrome P-450 (at -180°). The viologen dyestuffs used in this work were purchased from Mann Research Labs. Benzyl viologen (lot R2829) had an E_o' of -0.316 volt at pH 7.0 and an extinction coefficient of 8.0 x 10^{-3} $M^{-1}cm^{-1}$ at 555 mu. Methyl viologen (lot S3534) had an E_o' of -0.440 volt at pH 7.0 and an extinction coefficient of 9.8 x 10^3 $M^{-1}cm^{-1}$ at 604.5 mu. The reduced viologens were found to obey Beer's law in the concentration range employed in this study, and appeared to be in their mon-

omeric forms according to the spectral criteria. Under certain conditions these viologens do not obey Beer's law (9).

RESULTS AND DISCUSSION

The percentages of microsomal cytochrome P-450 which were reduced at various potentials established by mixtures of oxidized and reduced viologens in the absence of oxygen are depicted in Fig. 1. It was not possible for us to establish potentials below -0.44 volt with the redox systems we employed.

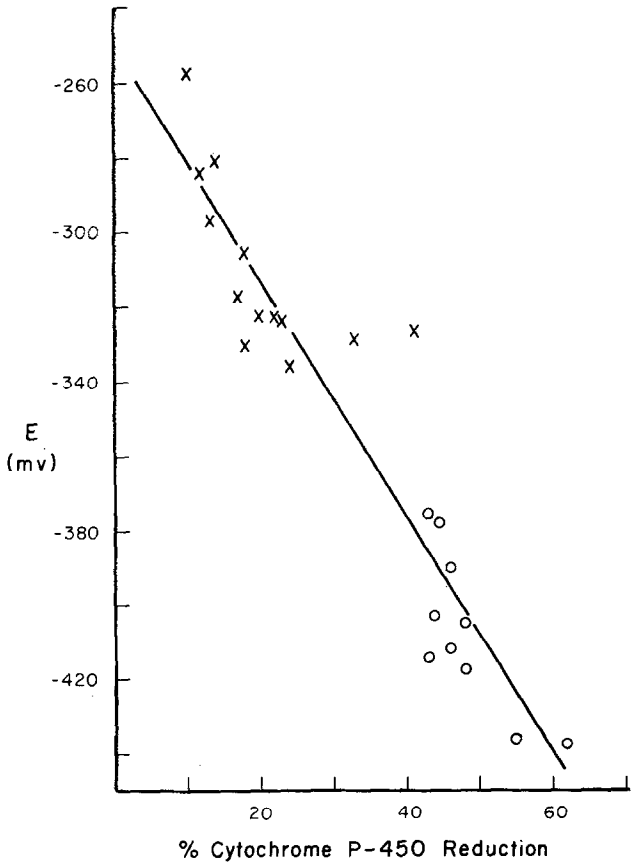

Fig. 1. Percentages of microsomal cytochrome P-450 reduced at various potentials. The ordinate is potential (E) in millivolts. The abscissa is per cent reduction of the g = 2.25 ESR peak of cytochrome P-450. x = points obtained with reduced benzyl viologen. o = points obtained with reduced methyl viologen. The solid line is fitted to the experimental points by the method of least squares. The microsome preparation used to obtain these points contained 18 mg. protein/ ml., 2.22 mumoles cytochrome P-450/ mg. protein, and was dispersed in 0.1 M phosphate buffer pH 7.0.

The titration extends only to 60% reduction, over a range of 0.18 volt, with 50% reduction occurring at about -0.41 volt. A line has been fitted to the experimental points by the method of least squares (Fig. 1). This representation is uninflected, and we can offer no hypothesis to explain it. In any case, it is clear from our results that about 50% of the cytochrome P-450 titrated in situ is reduced at -0.41 volt. This is a very low value for a hemoprotein.

The complex spectral and redox properties of whole microsomes made it impossible for us to measure all the relevant concentrations of viologens and cytochrome P-450 at the same temperature, with presently available techniques. Consequently, the ratios of oxidized to reduced viologen were determined at room temperature, and the ratio of oxidized to reduced cytochrome P-450 (microsomal Fe_x) was measured at -180°. It is probable that the redox systems involved in the measurement have some temperature dependency; however, the redox system, at equilibrium, was rapidly cooled in liquid nitrogen, and we assume that no significant redox shift occurred among the components of the system during the time in which they cooled from 25° to the solid, reactively sluggish, frozen state.

The oxidation-reduction potential of cytochrome P-450 is the lowest reported for any cytochrome in its native environment. The question of how this low redox requirement is met by NADPH is an interesting one. Certainly the potential of NADPH in the microsomal membrane is unknown and might be low enough to meet these requirements. Liver NADPH-cytochrome c reductase is thought to be the electron donor for cytochrome P-450 and has been shown to alternate between the fully-reduced and half-reduced forms during catalysis (10). Recently it has been shown that the E'_o of the P. elsdenii reduced flavodoxin/flavodoxin semiquinone couple is -0.371 volt at pH 7.0 (11). This suggests that the NADPH-cytochrome c reductase catalytic mechanism could fit the cyto-

chrome P-450 redox requirements in the microsomes. A further possibility is that the redox potential of cytochrome P-450 is raised by combination of this heme protein with substrate during mixed function oxidation reactions. A body of evidence has been accumulated which indicates that substrate binds to oxidized cytochrome P-450 (12).

Cytochrome P-450 is involved in mixed function oxidation reactions and is very autoxidizable. The redox potential required to reduce molecular oxygen to the superoxide anion (O_2^-) in a one-equivalent process lies between -0.45 and -0.3 volt at pH 7 (13, 14); consequently, the autoxidation of cytochrome P-450, if it occurs by a one-equivalent process, could produce the superoxide anion.

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