Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 353-360

FLUORESCENT ENERGY TRANSFER MEASUREMENTS ON FLUORESCEIN ISOTHIOCYANATE MODIFIED CYTOCHROME P-450 LM2

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Received April 18, 1983

SUMMARY: The distance between the heme iron and the N-terminus of cytochrome P-450 LM2 was determined by fluorescence energy transfer measurements. Fluorescein isothiocyanate which was covalently bound to the N-terminal methionine was used as donor chromophor. The R_o value between fluorescein isothiocyanate and the heme was calculated to be 3.98 nm. The distance between the nitrogen of the N-terminal methionine and the heme was estimated with 2.84 ± 0.23 nm excluding most likely the N-terminal amino acid of cytochrome P-450 LM2 to participate in the electron transfer to the heme iron. A cytochrome P-450 LM2 membrane model is proposed.

The mixed function oxidase of the hepatic endoplasmic reticulum (EC 1.14.14.1) is characterized by its capability to activate molecular oxygen. This enzymatic process is based on the insertion of electrons into the oxygen molecule provided by the NADPH-dependent P-450 reductase (EC 1.6.4.2). These two proteins are functionally linked via the phospholipid bilayer matrix to enable electron transfer, substrate and oxygen binding as well as oxygen activation and hydroxylation. A thorough understanding of the multistep reaction mechanism needs detailed knowledge of the structural requirements. Besides the active center the interacting region and the electron pathway are structural essentials for the catalytic function.

Selective chemical modification has proved as appropriate means to identify amino acid residues either involved in interac-

Abbreviations: cytochrome P-450 LM2 = P-450 LM2
NADPH-cytochrome P-450 reductase = reductase
fluorescein isothiocyanate = FITC

0006-291X/83 $1.50

tions between reductase and P-450 /1/ or localized in the vicinity of the heme iron /2/. Experimentally proved data about the way the electron passed from the flavins of the reductase to the heme iron, however, are rather poor.

An experimental approach to better understand the structural details which enable the electron transfer represents the determination of distances between functionally important sites. The determination of energy transfer of fluorescent chromophores to acceptor molecules according to Förster /3,4/ has proved suitable to study such long-range interactions. Recently this method has been successfully applied to study interactions between cytochrome c and adrenodoxin and to study the cytochrome c/cytochrome b_5 arrangement /5,6/.

The present paper is aimed to decide if the interacting region of P-450 LM2 with reductase, which involves the N-terminal methionine, simultaneously can be regarded as the entrance of the pathway the electrons are passing to the heme iron.

MATERIALS AND METHODS

P-450 LM2 was isolated from liver microsomes of phenobarbital-pretreated male rabbits according to /7/ and labeled with FITC isomer 1 by incubation at pH 7.4 and 277 K for 1 h according to /1/. The unreacted FITC was removed by passage through a Sephadex G-25 column. The bound FITC was determined by use of $\epsilon_{496\ nm}$ = 74.5 $mM^{-1} cm^{-1}$ and the heme to protein ratio was calculated on the basis of the ratio A_{417}/A_{278}. The spectrophotometric measurements were carried out on a Shimadzu UV-VIS 300, Japan. Emission and excitation wavelength corrected fluorescence spectra and fluorescence polarizations were recorded on the fluorescence spectrophotometer Perkin-Elmer MPF 44B. Quantum yields were determined utilizing fluorescein in 0.1 N NaOH (q = 0.92 /8/) as fluorescence standard.

RESULTS AND DISCUSSION

Energy transfer measurements require the overlap of the fluorescence spectrum of a donor group with the absorption spectrum of an acceptor group.

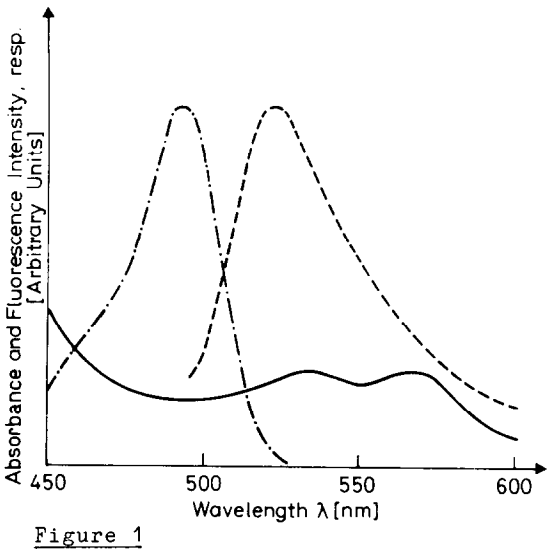

Figure 1

Spectra of FITC and P-450 LM2:
——— absorption of P-450 LM2
----- fluorescence of FITC-P-450 LM2
-.-.- absorption of FITC

Fig. 1 shows the fluorescence and absorption spectra of the donor and acceptor group, respectively. The spectral overlap of FITC fluorescence band with the heme absorption Q-bands is demonstrated thus accomplishing a necessary prerequisite for energy transfer measurements.

The energy transfer efficiency E is described by the Förster formula /3,4/:

$$E = 1-F/F_o = 1/(1 + (R/R_o))^6 \qquad (1)$$

with

$$R_o = (8.79 \cdot 10^{-25} \cdot q \cdot k^2 \cdot n^{-4} \cdot J)^{1/6} \ [cm] \qquad (2)$$

F_o = fluorescence intensity without acceptor, F = fluorescence intensitiy with acceptor, R = mean distance between the chromophores, R_o = critical transfer distance indicating an energy transfer efficiency of 50 % q = quantum yield without acceptor, k = orientation factor, n = refractive index of the protein solution, J = spectral overlap integral of donor emission and acceptor absorption according to

$$J = \frac{\int F(\lambda) \cdot \epsilon(\lambda) \cdot \lambda^4 d\lambda}{\int F(\lambda)\, d\lambda} \qquad (3)$$

with λ = wavelength and ϵ = absorption coefficient.

In the case of heme enzymes with heme as acceptor the fluorescence of apoenzyme (F_o) and holoenzyme (F) should be determined. Due to poor availability of pure apo- and holoenzyme the respective fluorescence intensities have been extrapolated from plotting the values of different P-450 LM2 samples with varying heme contents.

In Fig. 2 the correlation of the quantum yields with the percentage of the heme to protein ratios are shown. The linear correlation (r = -0.95) enables the extrapolation to the heme free apo- and the heme containing holoenzyme with FITC quantum yields of q_{apo} = 0.676 and of q_{holo} = 0.138, respectively. Based on these values E has been calculated to E = 0.796. Fluorescence polarizations are not higher than 0.08...0.12 for all FITC P-450 LM2 preparations and the quantum yield of FITC in buffer solution q_{FITC} = 0.67 is equal to q_{apo}. Therefore a predominant statistical orientation can be assumed with a k^2 of 2/3 /9/. The overlap

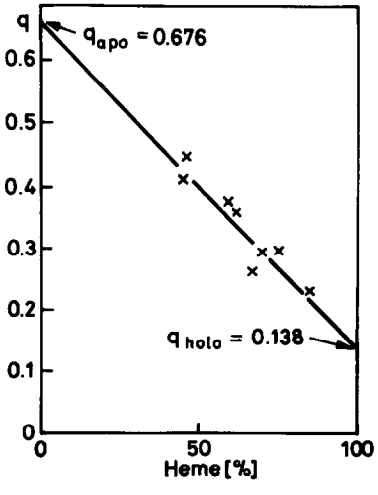

Figure 2

FITC fluorescence quantum yields in dependence on the heme contents (correlation coefficient -0.95, standard deviation 0.08)

integral calculated from the spectra according to equation (3) revealed a value R_o = 3.98 nm (with n = 1.4) from which a mean distance between the heme and the FITC molecule of R = 3.17 nm was calculated. Because of the limitation for an exact estimation of the orientation factor k an error of 20 % for any given possible fixed orientation of the chromophores is usually given /6/.

In our case of statistical arrangement and multiple transition dipole moment orientations the error would be reduced to about 8 %.

In order to decide if the N-terminus directly participates in the electron transfer or if its functional importance /1/ rather is related to mediate P-450/reductase interactions the shortest possible distance between the N-terminus and the heme is of interest. The binding site for the FITC residue at the N-terminus (isothiocyanate group) however is not identical with the fluorescence group. Even if one considers that the electron may be transferred to the iron via the porphyrin π -system the distance is shortened only by - 0.92 nm thus only slightly modifying the result. This range of correction was calculated by use of the increment method for binding values for any possible orientation between both chromophores independent of steric hindrance of any FITC position caused by the protein environment. A detailed geometric analysis of this problem includes a probability weighting of the FITC arrangements. Considering these most probable positions and the error of 8 % due to the orientation factor used a value for the distance between the midpoints of the xanthene ring and the porphyrin iron of R = 2.84 $\pm$ 0.23 nm is calculated. Because the estimated R is the mean of distances between all FITC molecules and heme groups of the P-450 LM2 oligomer /10/ an intermolecular energy trans fer cannot be excluded. Measurements on monomeric P-450 LM2 in the presence of detergents (sodiumcholate, octyl-ß-D-glucopyranoside,

Triton N-101, and Lubrol PX) proved the intermolecular contribution to be small. Therefore this contribution would be not interfere with the calculated distance.

It can be assumed that in a macromolecular scale the shape of P-450 LM2 in solution does not deviate profoundly from that in the microsomal membrane despite experimental findings evidencing distinct individual conformations of both states /11, 7/, see Table 1. Based on this assumption and on data concerning the geometry of P-450 LM2 the distance determined on the soluble form can be extrapolated to P-450 LM2 in the microsomal membrane. The results obtained on microsomal P-450 further allow to localize heme and the N-terminus relative to the membrane: the heme is $\leq$ 2 nm apart from the surface and oriented within the membrane parallel to the surface /15/. The N-terminus is at the cytosolic leaflet and is readily accessible /1/. With an approximated protein radius of 2.1 nm and an immersion depth of 2.3 nm /12/ we obtain the following model for membrane-bound P-450 LM2 (membrane data: egg lecithin, sonicated /16/), see figure 3. It shows the possible arrangements of the N-terminus in dependence on the different heme locations in the membrane. Due to the inaccessibility of the heme for the substrate position C is unlikely, and therefore an arrangement of catalytic important groups according to a position between A and B is to be expected.

The estimated minimal distance between the N-terminal methionine and heme of 2.61 nm is too large to be surmounted by a thermally activated tunneling mechanism. The latter would be able to bridge a distance of maximally 1.0 nm /17/. Thus it may be concluded that the N-terminus of P-450 LM2 which is involved in interactions with the reductase most likely does not participate in the electron transfer pathway from the NADPH to the FMN via FAD of the reductase to the heme iron.

TABLE 1

Estimation of oligomeric volumina and monomeric radii of P-450 LM2 from different experimental methods (a_{exp} = rotameric radius of the hexamer, h_{exp} = immersion depth of the rotamer in the membrane, $\mathcal{R}$ = spheric radius of the oligomer, r = radius of the monomer, V = volume of the oligomer)

Method	Medium	Supposed geometry	a_{exp} /nm/	h_{exp} /nm/	$\mathcal{R}_{exp}$ /nm/	r /nm/	V /nm^3/	Ref.
Time-dependent polarized emission of fluorescence	Membrane	Rotamer (torus)	6.4	2.3		$\frac{2a \sin 15°}{1+2\sin 15°} = 2.18$ $h = 2.3$	$2\pi^2(a-r)r^2 = 396$ $2\pi^2(a-h)h^2 = 428$	/12/
Hydrodynamical measurement	Solution	Most dense packing of spheres			4.59	$\frac{\mathcal{R}\cos 30°}{1+\cos 30°}\Big/\sqrt{1+\left(\frac{\sin 30°}{1+\sin 30°}\right)^2} = 2.02$	$\frac{4}{3}\pi\mathcal{R}^3 = 409$	/13/
Fluctuation spectroscopy	Solution	Most dense packing of spheres			4.86	$\frac{\mathcal{R}\cos 30°}{1+\cos 30°}\Big/\sqrt{1+\left(\frac{\sin 30°}{1+\sin 30°}\right)^2} = 2.14$	$\frac{4}{3}\pi\mathcal{R}^3 = 480$	/14/

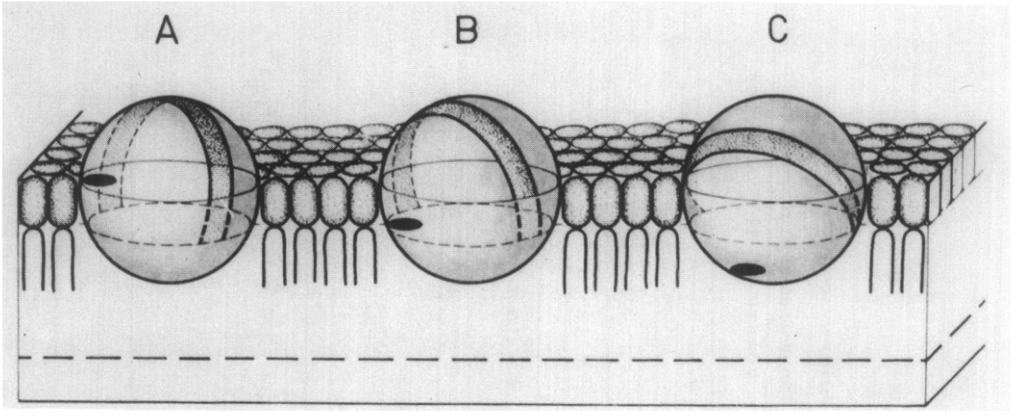

Figure 3 Localization of the N-terminal amino acid of membrane bound cytochrome P-450 LM2, showed by shaded area (Protein position within the membrane according /12/ with 3 heme positions within the range proposed by Rich /15/: The heme (full discs) is assumed in A: on the membrane surface. B: in the depth of 1 nm, C: in the depth of 2 nm)

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