Identification of the ligand *trans* to thiolate in cytochrome P-450 LM2 by chemical modification

G.-R. Jänig, R. Dettmer*, S.A. Usanov+ and K. Ruckpaul

*Department of Biocatalysis and *Department of Analytic and Biotechnology, Central Institute of Molecular Biology, GDR Academy of Sciences, 1115 Berlin-Buch, GDR* and *+Institute of Bioorganic Chemistry, Belorussian Academy of Sciences, Minsk 220600, USSR*

Received 10 May 1983

About 3 tyrosine residues of cytochrome P-450 LM2 are accessible to chemical modification with tetranitromethane. Nitration of two tyrosines inactivates the enzyme to about 20%. The partial formation of a hyper-porphyrin spectrum originating from the pK shift by nitration and formation of a tyrosinate is prevented by modification in the presence of the inhibitor metyrapone. These findings support the assumption of a tyrosine residue as sixth ligand of the heme iron in cytochrome P-450 LM2.

Cytochrome P-450 *Active center* *Sixth ligand* *Tyrosine* *Chemical modification*

1. INTRODUCTION

The catalytic function of cytochrome P-450 (P-450, EC 1.14.14.1) is based on its capability to activate molecular oxygen by inserting electrons which are supplied to the heme iron by NADPH via a flavoprotein. Essential for this process are the strong electron-donating properties of an experimentally evidenced thiolate group as fifth heme iron ligand [1]. Contrary to the fifth ligand, the nature of the sixth heme iron ligand is less defined. Recent experimental evidence is accumulating that oxygen may be the axial ligand *trans* to the thiolate group [2,3]. However, it is an open question as yet to what amino acid residue this oxygen can be attributed.

Chemical modification in general has proved an appropriate tool to determine the functional importance of distinguished amino acid residues. Respective studies on the phenobarbital-inducible isozyme P-450 LM2 have been directed either to define groups essential for protein–protein interactions [4] or to characterize residues which participate in the electron pathway or are involved in the formation of the active center [5]. Based on preceding studies [6] this report deals with the selective modification of tyrosine in P-450 LM2 with tetranitromethane (TNM). The results provide further experimental support for the assumption that tyrosine functions as sixth heme iron ligand.

2. MATERIALS AND METHODS

2.1. *Materials*

P-450 LM2 was isolated from phenobarbital-pretreated male rabbits according to the published procedure [7] with specific contents from 14.8 to 17.3 nmol P-450/mg protein. Metyrapone (1,2-di-(3-pyridyl)-2-methyl-1-propanone) and TNM were purchased from Serva (Heidelberg). All other materials were of analytical reagent grade.

2.2. *Nitration of P-450 LM2*

Nitration of the enzyme with TNM [8,9] was performed at 25°C in 1.5 ml reaction mixtures consisting of about 30 μM P-450 LM2 (heme basis) in 0.05 M Tris–HCl (pH 7.5), 20% (w/v) glycerol, and a 5–50-fold molar excess of TNM over enzyme corresponding to a maximal concentration of

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

about 1.5 mM TNM. Up to 30 μl of an 84 mM ethanolic solution of TNM were added to the reaction mixture. The excess of the reagent and the nitroformate ion were removed after 30 min from the nitrated enzyme by passing the 1.5 ml reaction mixtures through Sephadex G-25 (fine) columns (1 × 12 cm), using 0.1 M potassium phosphate, 20% (w/v) glycerol (pH 7.5) as buffer for equilibration and elution.

2.3. *Determination of the extent of nitration*

The 3-nitrotyrosine content of P-450 LM2 samples modified by different concentrations of TNM was determined by amino acid analysis on an amino acid analyzer (AAA 881 Mikrotechna, Prague). The dialyzed freeze-dried samples were hydrolyzed in 6 N HCl at 110°C for 24 h. For prevention of possible oxidation of tyrosine during hydrolysis, 1% phenol was added to the samples.

Due to better spectral resolution of overlapping bands and shoulders [10,11] second derivative spectroscopy was additionally used to quantitate the extent of nitration. Nitration of P-450 LM2 decreases the difference between the peak minimum at 283 nm and the peak maximum at 287 nm which is characteristic of tyrosine in the second derivative spectrum [6]. The 3-nitrotyrosine formed on nitration shows a negligible absorption difference at these wavelengths (spectra not shown). The decrease in the peak to trough difference related to a control with the same protein concentration correlates linearly with the increasing content of 3-nitrotyrosine in P-450 LM2 determined by amino acid analysis.

2.4. *Analytical methods*

Absorption spectra, together with the corresponding derivative spectra, were measured using a dual wavelength UV-300 spectrophotometer (Shimadzu, Kyoto) with the DES-1 derivative spectrophotometric accessory unit.

The concentrations of P-450 and P-420 were calculated from carbon monoxide difference spectra [12]. The determination of sulfhydryl groups was carried out with 5,5′-dithio-bis(2-nitrobenzoic acid) taking an absorption coefficient of 13.6 $mM^{-1}.cm^{-1}$ [13] for the increase of absorbance at 418 nm.

The benzphetamine *N*-demethylase activity of the modified P-450 LM2 samples was determined after reconstitution with NADPH–cytochrome P-450 reductase and L-α-dilauroylphosphatidylcholine (DLPC) as in [14].

3. RESULTS AND DISCUSSION

TNM has been reported as a relatively specific reagent for the nitration of tyrosine residues under mild conditions [15]. The amount of nitrated tyrosine residues increases with the TNM/P-450 LM2 ratio as indicated in table 1. A 10-fold molar excess results in the introduction of about 1.3 nitro groups into the enzyme, whereas about 2 tyrosine residues are nitrated at a 20-fold excess. More than 3 tyrosine residues are modified with a 30-fold excess, but no more than 3.6 tyrosines/enzyme molecule can be nitrated at a 50-fold molar excess of TNM. Therefore, the residual 7–8 tyrosines of P-450 LM2 out of a total of 11 present in our P-450 LM2 samples, are not accessible to modification and should be located in buried positions. The number of exposed tyrosine residues accessible to modification is in good accordance with the results of pH-titration [6] and acetylation [16].

As shown in fig.1 the nitration of tyrosine results in a lowered *N*-demethylase activity. Nitration of up to 2 tyrosine residues inactivates almost linearly the initial enzymatic activity to about

Table 1

Nitration of cytochrome P-450 LM2 by tetranitromethane

Molar excess over P-450	3-Nitrotyrosine residues (mol/mol P-450)	
	without metyrapone	with metyrapone
10	1.36 ± 0.26 [4]	n.d.
20	1.96 ± 0.19 [6]	0.62 ± 0.02 [3]
30	3.14	1.39
50	3.61	–

n.d., not detectable

Treatment for 30 min at 25°C in 50 mM Tris–HCl buffer, 20% (w/v) glycerol (pH 7.5). The excess of the reagent and the nitroformate formed were removed by gel filtration on Sephadex G-25. For protection against nitration, samples were preincubated with 4 mM metyrapone before the addition of tetranitromethane

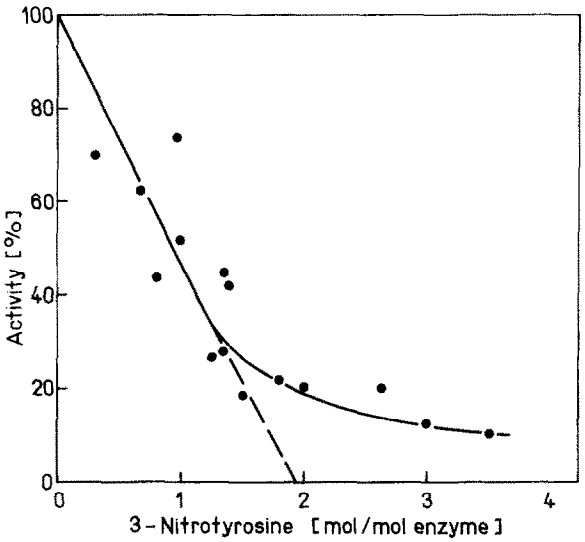

Fig.1. Relationship between the inactivation of cytochrome P-450 LM2 and the number of tyrosine residues modified by tetranitromethane. Benzphetamine *N*-demethylase activity was measured after reconstitution with the following ratio of the components: 1 nmol P-450, 0.05 nmol reductase, 32 nmol D-α-dilauroyl phosphatidylcholine (100% control activity = 7.23 ± 0.79 nmol HCHO.min^{-1}. mol P-450^{-1}, n = 9). The activities refer to those of a control with the same protein concentration including the portion of the CO-unreactive material.

20%. Further modification leads to an almost complete loss of activity. This result indicates an essential functional importance of two accessible distinct tyrosine residues.

In addition to nitration of tyrosine, a modification of tryptophan and oxidation of sulfhydryl groups by TNM are known as unwanted side reactions [15]. A reaction of tryptophan which would be very slow under the conditions employed can be excluded due to an absent absorption near 330 nm characteristic of 7-nitroindole derivatives [17]. Further support against tryptophan nitration, which is insensitive to sodium dithionite [17], can be derived from complete sensitivity to sodium dithionite which was evidenced by high-pressure liquid chromatography analysis of tryptic peptides of correspondingly treated P-450 LM2 samples (unpublished). A reaction of cysteine with TNM would generate a mixture of disulfide and sulfinic acid [15]. The number of accessible sulfhydryl groups in modified P-450 LM2 samples was measured by titration with 5,5'-dithio-bis-(2-nitrobenzoic acid) to determine the extent of such a side reaction. The gradual decrease of the number of reactive SH-groups (1.06 ± 0.10, n = 5) in the course of nitration which is paralleled by an increase in the content of cysteic acid indicates the occurrence of an oxidation reaction. However, a functional importance of the one accessible SH-group of P-450 LM2 was previously excluded by modification experiments using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole [18]. Thus, most likely the decreased enzymatic activity of nitrated P-450 LM2 can be attributed mainly to the modification of tyrosyl residues.

This loss is partially caused by a diminished amount of catalytically active P-450. Increasing concentrations of TNM lead to an enhanced conversion of P-450 LM2 into a form which is unable to bind carbon monoxide after reduction by sodium dithionite. At a 10-fold excess of TNM, about 70–80% of P-450 LM2 still reacts with CO including the enzymatically inactive cytochrome P-420 which amounts to up to 15% under these conditions. A 20-fold excess diminishes the CO-binding capability of P-450 LM2 to 30–40% without further increasing the P-420 content. These findings indicate that the heme environment of P-450 LM2 is affected by nitration, also indicating a location of tyrosine residues in this area.

The nitration of tyrosine residues results in a pK shift of the phenolic hydroxyl group from 9.8 to about 7.0 [15]. Thus, at pH 7.5, the major portion of the 3-nitrotyrosine residues will be present as negatively charged nitrotyrosinate ions. The conversion of a neutral tyrosine into a charged nitrotyrosinate is accompanied by changes in the UV-visible absorption spectrum of ferric P-450 LM2 as shown in fig.2. With increasing molar excess of TNM the nitrotyrosine absorbance at 278 nm increases, whereas that in the Soret region decreases concomitant with a broadening of this band and a shift to shorter wavelengths (418 nm to 414 nm). On the other hand, the absorbance at 377 nm and at 465 nm increases considerably. Moreover the nitrated P-450 LM2 exhibits a more intense β-band than α-band ($A_{534} > A_{568}$) as compared with the native enzyme. These spectral shifts clearly demonstrate that the axial symmetry of the heme in P-450 LM2 changes on nitration by TNM.

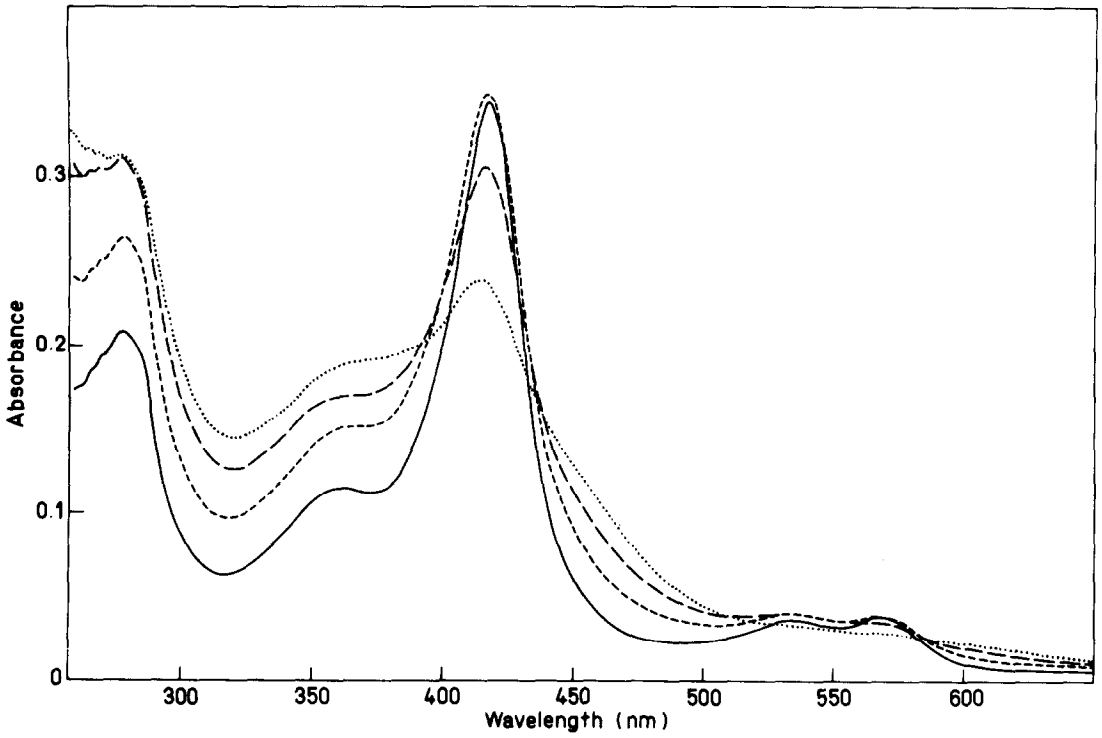

Fig.2. UV-visible absorption spectra of ferric cytochrome P-450 LM2 modified by tetranitromethane. Experimental conditions: cytochrome P-450 LM2, 6.3 μM; 0.1 M potassium phosphate buffer, 20% (w/v) glycerol (pH 7.5); light path, 0.5 cm; room temperature; control (——), P-450 LM2 modified by 9.6-fold (---), 19.3-fold (– – –), and 31.1-fold (···) molar excess of TNM.

The resulting peak and shoulder at 377 nm and at 465 nm, respectively, as well as the broad Q-band absorption indicate the partial formation of a socalled hyper-porphyrin spectrum (spectrum with a split Soret band) in the presence of two partially negatively charged axial ligands of the heme iron (thiolate and tyrosinate) with 3 p-orbitals overlapping with the iron d-orbitals [19]. Thus, the complicated observed UV-visible spectrum represents a superposition of 3 spectra: a mixture of modified P-450 either with a nitrophenol or nitrophenolate residue, and native P-450 LM2. Therefore, the partial formation of a hyper-porphyrin spectrum provides first experimental evidence that the oxygen atom of the second axial ligand postulated by various investigators [2,3] may come from a tyrosine residue in P-450 LM2.

To prove the axial symmetry of the heme iron EPR-spectra of the nitrated P-450, LM2 were measured. The obtained g-values of 1.94, 2.26 and 2.43 are characteristic of a ferric low-spin P-450 [3]. The measured g-values therefore exclude any changes of the fifth heme iron ligand at nitration of P-450 LM2. However, the unchanged EPR-spectrum of the nitrated protein indicates that also the sixth ligand is not displaced by another atom; e.g., nitrogen. In such a case the g_3-value should be greater than 2.46 [3].

In order to further experimentally support the assumption of a tyrosine residue within the active center of P-450 LM2 as sixth heme iron ligand, nitration was performed in the presence of the inhibitor metyrapone which is coordinated via one of its pyridine nitrogens to the heme iron of P-450 [20]. Modification with a 20-fold and 30-fold molar excess of TNM in the presence of metyrapone results in a lowered extent of nitration (table 1). A competition of metyrapone with P-450 LM2 for TNM as the reason for the decreased modification was excluded. At least one tyrosine is protected from nitration by the inhibitor metyrapone supporting the location of one

tyrosine in the immediate vicinity of the heme iron.

The molecular parameters of metyrapone are sufficient to prevent nitration of a tyrosine residue within a maximal distance of 13 Å between the phenolic group of tyrosine and the heme group. The absence of a hyper-porphyrin spectrum in the presence of metyrapone is in agreement with the existence of tyrosine as a sixth heme iron ligand. Further support was obtained by converting the nitro group into an amino group with $Na_2S_2O_4$ which raises back the pK of the phenolic hydroxyl group to about 10 [15]. The disappearance of the spectral changes observed after nitration also indicates that the presence of a negatively charged nitrotyrosinate ion is essential for the hyper-porphyrin spectrum.

From the data presented it is concluded that in P-450 LM2 a tyrosine is located in such a position which allows direct interaction with the heme iron, thus acting as axial heme iron ligand *trans* to thiolate.

ACKNOWLEDGEMENTS

The expert technical assistance of Mrs H. Grill is gratefully appreciated. We would like to thank Dr O. Ristau (Department of Hemecatalysis, Central Institute of Molecular Biology) for performing the EPR measurements.

REFERENCES

[1] Champion, P.M., Stallard, B.R., Wagner, G.C. and Gunsalus, I.C. (1982) J. Am. Chem. Soc. 104, 5469–5472.

[2] White, R.E. and Coon, M.J. (1982) J. Biol. Chem. 257, 3073–3083.

[3] Dawson, J.U., Andersson, L.A. and Sono, M. (1982) J. Biol. Chem. 257, 3606–3617.

[4] Bernhardt, R., Etzold, G., Stiel, H., Schwarze, W., Jänig, G.-R. and Ruckpaul, K. (1982) in: Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications (Hietanen, E., Laitinen, M. and Hänninen, O. eds) pp.581–584, Elsevier, Amsterdam, New York.

[5] Dus, K. (1980) in: Biochemistry, Biophysics and Regulation of Cytochrome P-450 (Gustafsson, J.-Å., Carlstedt-Duke, J., Mode, A. and Rafter, J. eds) pp.129–132, Elsevier, Amsterdam, New York.

[6] Ruckpaul, K., Rein, H., Ballou, D.P. and Coon, M.J. (1980) Biochim. Biophys. Acta 626, 41–56.

[7] Haugen, D.A. and Coon, M.J. (1976) J. Biol. Chem. 251, 7929–7939.

[8] Bruice, T.C., Gregory, M.J. and Walter, S.L. (1968) J. Am. Chem. Soc. 90, 1612–1619.

[9] Sokolovsky, M., Riordan, J.F. and Vallee, B.L. (1966) Biochemistry 5, 3582–3589.

[10] Balestrieri, C., Colonna, G., Giovane, A., Irace, G. and Servillo, L. (1978) Eur. J. Biochem. 90, 433–440.

[11] Ruckpaul, K. and Rein, H. (1980) Acta Biol. Med. Germ. 39, 55–69.

[12] Omura, T. and Sato, R. (1967) Methods Enzymol. 10, 556–561.

[13] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.

[14] Jänig, G.-R., Pfeil, D. and Ruckpaul, K. (1979) Acta Biol. Med. Germ. 38, 409–422.

[15] Riordan, J.F. and Vallee, B.L. (1972) Methods Enzymol. 25, 515–521.

[16] Jänig, G.-R., Smettan, G., Friedrich, J., Bernhardt, R., Ristau, O. and Ruckpaul, K. (1982) in: Cytochrome P-450 Biochemistry, Biophysics and Environmental Implications (Hietanen, E. et al. eds) pp.513–516, Elsevier, Amsterdam, New York.

[17] Sokolovsky, M., Fuchs, M. and Riordan, J.F. (1970) FEBS Lett. 7, 167–170.

[18] Ngoc Dao, N.T. (1981) Doctoral Thesis, GDR Academy of Sciences, Berlin.

[19] Jung, Ch. (1983) Stud. Biophys., in press.

[20] Ruf, H.H. and Nastainczyk, W. (1976) Eur. J. Biochem. 66, 139–146.