Volume 260, number 2, 289–290 FEB 08074 January 1990

The cosubstrate NADP(H) protects lysine 601 in the porcine NADPH-cytochrome P-450 reductase against pyridoxylation

Karin Müller, Dietmar Linder and Ludwig Lumper

Biochemisches Institut (Fachbereich Humanmedizin), Justus-Liebig-Universität, Friedrichstrasse 24, D-6300 Giessen, FRG

Received 27 November 1989

Lys[601] in NADPH-cytochrome P-450 reductase is modified by reductive alkylation with pyridoxal 5'-phosphate (pyridoxylation). Lys[601] is protected against modification by the cosubstrate NADP(H).

NADP(H) binding site; Lysine residue; Pyridoxal 5'-phosphate; Micro sequencing; (Pig liver)

1. INTRODUCTION

According to the model proposed for the arrangement of the nucleotide binding sites in P450R, the NADP-PP_i was tentatively assigned to the sequence Gly^{482}-Arg^{518} showing homology to FAD-proteins [1,2]. However, this identification of the NADP-PP_i site is not consistent with the following results: (i) the stretch Gly^{531}-Val^{536} present in all P450R with known structure [3,4] is related to a nucleotide (pyro)phosphoryl consensus sequence [2,5]; (ii) the P450R from yeast [3] shows insertions within the sequence corresponding to Tyr^{476}-Val^{507} representing the predicted NADP-PP_i site of mammalian P450R and (iii) the NADP(H)-protected Cys^{471} and Cys^{565} [2,6] are located outside of this sequence [2]. To get further information on the NADP(H) binding site, we identified the cosubstrate protected lysine(s) shown by differential labelling [8,9].

2. MATERIALS AND METHODS

2.1. *Purification of P450R*

Protease-solubilized P450R was isolated according to [10]. Enzymatic activity was measured at 25°C according to [10].

2.2. *Modification of P450R*

S-cyanylation and subsequent pyridoxylation of P450R was carried out at 25°C as follows: (i) P450R (40–60 μM) was reacted with 1 mM DTNB, 0.1 M NH_4HCO_3 pH 8.2 until 3 mol TNB^-/mol P450R were released. Reagents were removed on Sephadex G-25 equilibrated with 0.1 M NH_4HCO_3. To the eluate containing P450R, KCN was added up to 20 mM. After release of 3 mol TNB^-/mol P450R, the solution was loaded on Sephadex G-25 equilibrated with 0.1 M sodium borate pH 8.5 and eluted with this buffer. (ii) $(CN)_3$P450R (40–60 μM) was reacted with 1 mM PALP in 0.1 M sodium borate pH 8.5 for 15 min and then treated for a further 30 min after addition of $NaCNBH_3$ up to 0.1 M. Removal of reagents was achieved on Sephadex G-25 by elution with 0.1 M NH_4HCO_3 pH 8.2.

2.3. *Isolation of pyridoxylated peptides*

Digestion of the pyridoxylated $(CN)_3$P450R was performed with TPCK-trypsin ($(CN)_3$P450R:trypsin = 25:1 w/w) for 41 h at 37°C in 0.1 M NH_4HCO_3 pH 8.2. Peptides were separated on HPLC column (0.46 cm × 25 cm) of Bakerbond C_{18} material (5 μm; 33 nm) using a 0–50% CH_3CN gradient in 0.1% aqueous CF_3COOH (time of gradient: 60 min; 35°C; flow: 0.7 ml/min). Rechromatography was done on a Pharmacia Mino RPC SC_{18}/C_2 (0.46 cm × 20 cm; 5 μm; 10 nm; 12.5–50% CH_3CN in 0.1% CF_3COOH; time of gradient: 60 min; 35°C; flow: 0.7 ml/min).

Sequencing was done with a Biosystems 477A sequencer equipped with a model 120A on-line PTH analyzer.

3. RESULTS AND DISCUSSION

Pyridoxylation was carried out with S-cyanylated P450R, since the reductive alkylation of proteins containing accessible cysteines like P450R is not specific for lysine residues. S-cyanylation at the three reactive cysteines of P450R (Cys^{471}, Cys^{565} and Cys^{644}) was achieved as reported [7,11]. This modification did not adversely affect the P450R, since the specific activity of $(CN)_3$P450R is about 5% higher than that of the unmodified enzyme. Therefore, the loss of activity caused by pyridoxylation of $(CN)_3$P450R can be followed by NADPH-mediated cytochrome *c* reduction [11].

$(CN)_3$P450R was strongly inactivated by pyridoxylation under the conditions used (fig.1). In contrast, reductive alkylation with pyridoxal (1 mM) shows only a slight effect (fig.1). In the presence of 3 mM $NADP^+$

Correspondence address: L. Lumper, Biochemisches Institut, Justus-Liebig-Universität, Friedrichstrasse 24, D-6300 Giessen, FRG

Abbreviations: P450R, NADPH-cytochrome, P-450 reductase protease-solubilized (EC 1.6.2.4); NADP-PP_i, NADP pyrophosphoryl binding site; $(CN)_3$ P450R, NADPH-cytochrome P-450 reductase S-cyanylated at the accessible cysteines; PALP, pyridoxal s'-phosphate; pyridoxylation, substitution of lysyl residues by reductive alkylation with PALP; DTNB, 5,5'-dithiobis(2-nitrobenzoate); TNB^-, 2-nitro-5-thiobenzoate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

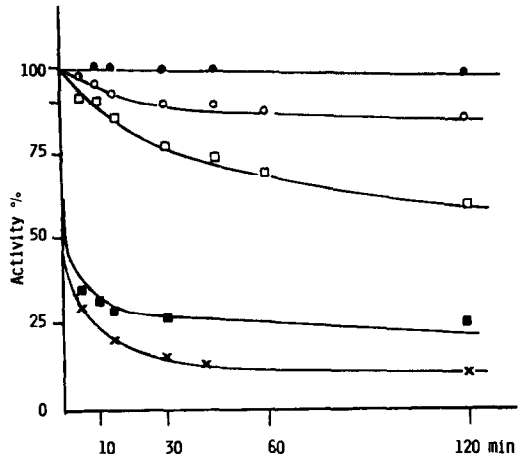

Fig.1. Modification of $(CN)_3$P450R by reductive alkylation with PALP. ●, control; X, + 1 mM PALP; □, + 1 mM PALP + 3 mM $NADP^+$; ■, 1 mM PALP + 3 mM NADH; ○, 1 mM pyridoxal.

(not NAD^+) inactivation by pyridoxylation is not abolished but its rate is decreased by a factor of about 5 (fig.1). This incomplete protection by a cosubstrate is likely to be caused by a slow modification of 'essential' lysines outside the NADPH binding site. If any, 10% of the total flavin is lost by P450R during pyridoxylation as determined by HPLC. To detect NADP(H)-protected lysine(s), $(CN)_3$P450R was pyridoxylated in the presence and absence of 3 mM $NADP^+$ for a period of 15 min (residual activity 85% and 20%, respectively) to avoid the reaction of lysines, whose reactivity is not dependent on the presence of cosubstrate. The HPLC map of the tryptic digest obtained from enzyme pyridoxylated in the presence of $NADP^+$ does not contain the main fluorescent peptide present in the HPLC map of the tryptic peptides from $(CN)_3$P450R pyridoxylated in the absence of cosubstrate (fig.2). Edman degradation of this peptide revealed the sequence **E-Q-P-Q-K^{601}-V-Y-V-Q-H-L-L-K^{609}**. At step 5 of the Edman degradation no PTH-lysine is released. Amino acid analysis of the peptide by determination of the phenylthiocarbamoyl derivates showed a substance eluted at a position typical for Nϵ-modified lysine. Cleavage of peptide E^{597}-K^{609} with chymotrypsin led to one fluorescent subpeptide with the sequence E^{597}-Y^{603}. Consequently, Lys^{601} is the site of pyridoxylation protected by $NADP^+$.

This result confirms the conclusion based on the protection of $Cys^{471}$and Cys^{565} by the cosubstrate that stretches outside the predicted NADP(H) binding region are involved in the binding of cosubstrate. In ferredoxin-$NADP^+$ oxidoreductase (spinach), Lys^{244} is labelled by oxidized $NADP^+$ [12]. This observation led to the suggestion that Lys^{601} of P45R located at a homologous position to that of Lys^{244} in the feredoxin-$NADP^+$ oxidoreductase is also participating in binding of NADP(H) [2,13]. Our studies confirm this proposal.

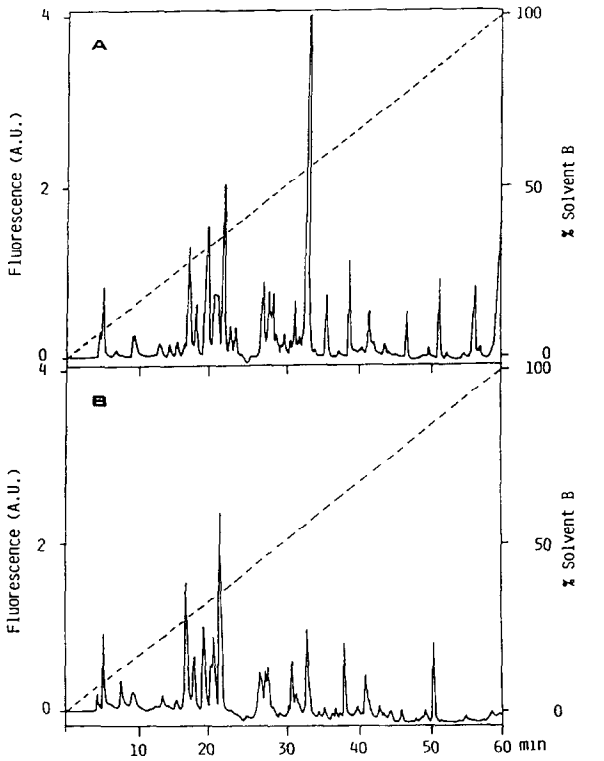

Fig.2. HPLC map of tryptic peptides obtained from $(CN)_3$P450R pyridoxylated in the absence (A) and the presence (B) of 3 mM $NADP^+$.

Acknowledgement: This work was supported by the Deutsche Forschungsgemeinschaft (Lu 149/11).

REFERENCES

[1] Porter, T.D. and Kasper, C.B. (1986) Biochemistry 25, 1682–1687.
[2] Vogel, F. and Lumper, L. (1986) Biochem. J. 236, 871–878.
[3] Yabusaki, Y., Murakami, H. and Ohkawa, H. (1988) J. Biochem. 103, 1004–1010.
[4] Ruettinger, R.T., Wen, L.-P. and Fulco, A.J. (1989) J. Biol. Chem. 264, 10987–10995.
[5] Katagiri, M., Murakami, H., Yabusaki, Y., Sugiyama, T., Okamoto, M., Yamano, T. and Ohkawa, H. (1986) J. Biochem. 100, 945–954.
[6] Haniu, M., Iyanagi, T., Legesse, K. and Shively, J.E. (1984) J. Biol. Chem. 259, 13703–13711.
[7] Vogel, F., Kaiser, C., Witt, I. and Lumper, L. (1985) Hoppe-Seyler's Z. Physiol. Chem. 366, 577–588.
[8] Inano, H. and Tamaoki, B. (1986) Eur. J. Biochem. 155, 485–489.
[9] Slepneva, I.A. and Weiner, L.M. (1988) Biochem. Biophys. Res. Commun. 155, 1026–1032.
[10] Vogel, F. and Lumper, L. (1983) Biochem. J. 215, 159–166.
[11] Lumper, L., Busch, F., Dzelic, S., Henning, J. and Lazar, T. (1980) Int. J. Pept. Protein Res. 16, 83–96.
[12] Chan, R.L., Carrillo, N. and Vallejos, R.H. (1985) Arch. Biochem. Biophys. 240, 172–177.
[13] Ostrowski, J., Barber, M.J., Rueger, D.C., Miller, B.E., Siegel, L.M. and Kredich, N.M. (1989) J. Biol. Chem. 264, 15796–15808.