The site of cyclic AMP-dependent protein kinase catalyzed phosphorylation of cytochrome P-450 LM2

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The phenobarbital-inducible form of cytochrome P-450 purified from rabbit liver microsomes is phosphorylated by cAMP-dependent protein kinase at a single site, the serine residue in position 128 of the amino acid sequence. The serine is located in a characteristic recognition sequence for cAMP-dependent protein kinase and is part of a primary structure which is conserved during evolution, present also in phenobarbital-inducible rat cytochrome and cytochrome P-450 CAM from *Pseudomonas putida*. The contribution of these findings to our understanding of the structure and membrane topology of cytochrome P-450 LM2 and its turnover regulated by phosphorylation is discussed.

Cytochrome P-450 *cAMP phosphokinase* *Protein phosphorylation*

1. INTRODUCTION

Protein phosphorylation is a regulatory principle in control of many cellular processes [1,2].

Phosphorylation of the phenobarbital-inducible form of cytochrome P-450 LM2 purified from rabbit liver microsomes in solution [3] and reconstituted membranes [4] by cAMP-dependent protein kinase was shown to decrease its enzymatic activity [4] by conversion into cytochrome P-420 with consequent loss of heme [5]. Thus breakdown of P-450 and therefore its turnover may be under the control of adrenergic transmitters mediated by the second messenger cAMP [5].

Here we show that P-450 is phosphorylated at a single site, the serine residue in position 128 of the amino acid sequence. This serine is located in a partial sequence which is specific for substrates of cAMP-dependent protein kinase. These findings contribute to our understanding of the structure and membrane topology of P-450 and regulation of P-450 turnover.

2. MATERIALS AND METHODS

[γ-^{32}P]ATP was purchased from Amersham, HPLC solvents from Baker, TPCK-trypsin from Merck, Darmstadt, and CM Sepharose C1-6B from Pharmacia, Freiburg i.Br. Reagents used for high-performance liquid chromatography (HPLC) were all of spectroscopic or HPLC grade. All other chemicals were of the highest quality commercially available.

Cytochrome P-450 LM2 was purified from liver microsomes of phenobarbital-treated rabbits by the method of Imai et al. [6]. These preparations had specific contents of 18–20 nmol/mg protein

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Abbreviations: P-450, cytochrome P-450 LM2; cAMP, 3′,5′-cyclic AMP; TCPK-trypsin, trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone; TFA, trifluoroacetic acid; cAMP kinase, catalytic subunit of cAMP-dependent protein kinase; PTH amino acids, phenylthiohydantoin amino acids

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and contained 0.2% Emulgen 913 and minute amounts of protein contaminants detectable by silver staining of their SDS electropherograms. The protein contaminants were removed before incubation by chromatography on a small aminooctyl Sepharose 4B column [6] equilibrated with 10 mM potassium phosphate buffer (pH 7.3) containing 20% glycerol and 0.2% Emulgen 913, the detergent by subsequent chromatography on CM Sepharose C1-6B.

The catalytic subunit of cAMP-dependent protein kinase was purified to homogeneity from bovine heart [7] to a specific activity of 3 units/mg.

Phosphorylation was performed in 1 ml of 50 mM Hepes buffer (pH 7.2) containing 1 mg cytochrome P-450, 70 ng catalytic subunit, 10 mM $MgCl_2$ and 25 μM [γ-^{32}P]ATP (500 cpm/pmol) at 30°C for 60 min. The reaction was stopped with TFA (final concentration 0.1%, v/v). The protein was separated from ATP by adsorbent filtration on a SEP-PAK C_{18} cartridge (Waters), eluted with 0.1% TFA/70% acetonitrile and lyophilized.

Purified and phosphorylated P-450 (5 mg) was cleaved by 0.1 mg TCPK-trypsin in 2 ml of 0.2 M ammonium bicarbonate (pH 8.3) at 37°C for 12 h, the reaction being stopped by adding 20 μl TFA. After lyophilisation the tryptic peptides were dissolved in 250 μl of 1% TFA containing 10% acetonitrile and separated by HPLC on a reversed phase C-18 wide-pore column (4.6 × 250 mm, pore size 300 Å, Vydac RP-218) using a Waters HPLC system; the chromatographic conditions, including those for rechromatography, are described in detail in the legends to figs 1 and 2. For detection, the UV absorbance at 214 and 280 nm was measured and ^{32}P radioactivity per fraction determined in parallel.

Rechromatographed pure peptides were subjected to amino acid analysis in a Durrum D-500 amino acid analyser after hydrolysis of the dried sample in 600 μl of 5.7 N HCl containing 0.5% phenol at 110°C for 24 h in evacuated tubes.

Edman degradation for amino acid sequence determination was done with a Beckman liquid phase sequencer with a spinning-cup modification in the presence of polybrene [8]. PTH derivatives were identified by HPLC and ^{32}P radioactivity of the degraded residues was measured by liquid scintillation counting using a Packard liquid scintillation counter.

3. RESULTS AND DISCUSSION

Comparison of the peptide profile of a gradient elution HPL-chromatogram of a tryptic digest of P-450 phosphorylated by cAMP kinase and the profile of ^{32}P radioactivity (fig.1) shows that almost all radioactive phosphate appears in one peak. Rechromatography of this peak under isocratic conditions separates the radioactive peptide from other nonradioactive peptides (fig.2). The radioactive peptide consists of 1 alanine, 2 arginines, 1 phenylalanine, 1 leucine, 1 methionine, 1 serine and 1 threonine as indicated by the amino acid analysis shown in table 1.

The sequence of these amino acids determined by Edman degradation is identical with the sequences of amino acids 126–133 of the known primary structure of rabbit cytochrome P-450 LM2 [9] and of rat cytochrome P-450 e [10], both

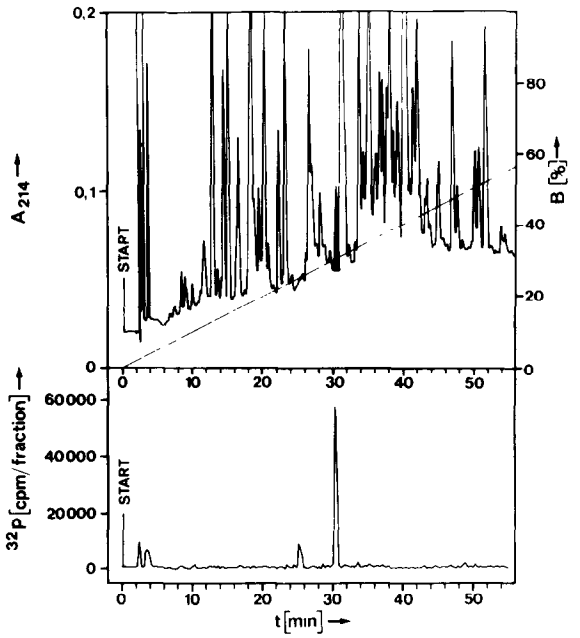

Fig.1. Gradient HPLC elution profile of the tryptic peptides of LM2 (1 mg injected) on a reversed phase C-18 column (4.6 × 250 mm, pore size 300 Å, Vydac RP-218); solvent A, 0.1% TFA; solvent B, 0.1% TFA/29.9% water/70% acetonitrile; flow rate 1.5 ml/min, 40°C. Upper panel: UV absorbance profile at 214 nm; the solid peak contains a radioactive tryptic peptide. The straight lines represent the gradient of solvent B in vol% (right ordinate scaling). Lower panel: ^{32}P radioactivity profile.

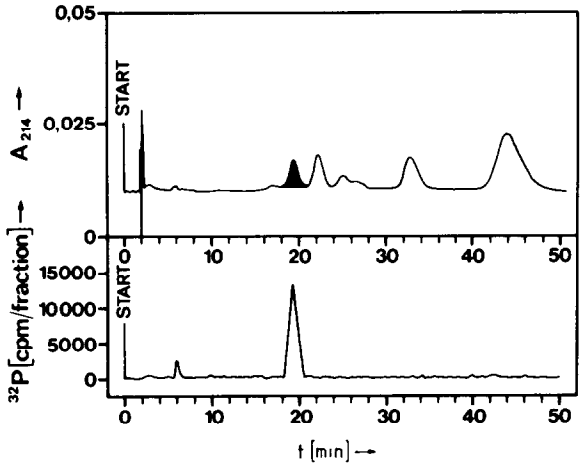

Fig.2. HPL rechromatography of the radioactive peak of fig.1 on the same column under isocratic conditions (80% solvent A, 20% solvent B). For other conditions see fig.1. Upper panel: UV absorbance profile at 214 nm, the filled peak contains the pure radioactive tryptic peptide. Lower panel: ^{32}P radioactivity profile.

isozymes inducible by phenobarbital (see fig.3). Furthermore, this is the only sequence in the primary structure of cytochrome P-450 LM2 which has the same amino acid composition as the radioactive peptide. Arg 126 is preceded by arginine and Arg 133 followed by aspartic acid. Both sequences are known cleavage sites for trypsin.

The site of phosphorylation is Ser 128. The radioactivity of PTH-serine released by Edman degradation rose to a peak 12-times the background intensity with a tail due to carry-over of radioactivity to subsequent cycles (fig.4).

Table 1

Amino acid composition of the phosphorylated peptide separated from a tryptic digest of cytochrome P-450 LM2

Amino acid	pmol	Residue
Alanine	495.1	1.13 (1)
Arginine	756.8	1.72 (2)
Leucine	546.2	1.24 (1)
Methionine	311.2	0.71 (1)
Phenylalanine	456.0	1.04 (1)
Serine	515.8	1.17 (1)
Threonine	439.1	1.00 (1)

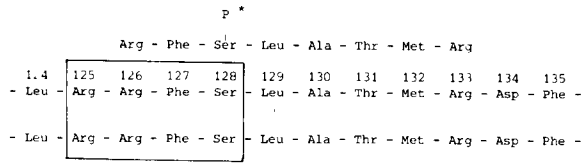

Fig.3. Comparison of the amino acid sequence of purified tryptic peptide (upper line), marked P* on serine for ^{32}P radioactivity, with published partial sequences of phenobarbital-induced cytochrome P-450 from rat liver [10] (middle line), and rabbit liver [9] (lower line). The rectangle induces the specific substrate sequence phosphorylated by cAMP-dependent protein kinase [12].

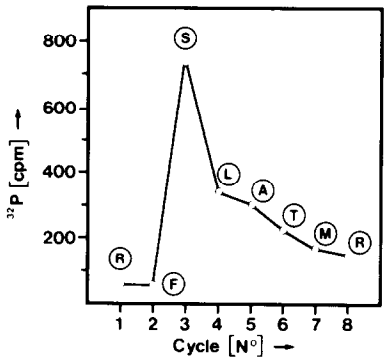

Fig.4. ^{32}P radioactivity of the PTH-amino acids released by sequential Edman degradation cycles.

The phosphorylation site is situated in a region of polar amino acids and close to the C-terminal end of one of the two regions of P-450 which are being discussed as part of the heme-binding site [9,11]. Both phosphorylation and heme reduction require access to these sites from the cytoplasm. That P-450 phosphorylation is observed in reconstituted membranes [4] is consistent with this requirement.

Our data support the view that specific phosphorylation of cytochrome P-450 LM2 and its ensuing transformation to cytochrome P-420 [5] which is considered to be an irreversibly denatured form has physiological significance as hormone-dependent 'down regulation' of the cellular level of P-450. The sequence Arg-Arg-Phe-Ser which is recognized by cAMP kinase [12] is present in the primary structure of rabbit cytochrome P-450 LM2 [9,13], rat cytochrome P-450 e [10] and cytochrome P-450 CAM from *Pseudomonas*

putida [14], but not in rabbit cytochrome P-450 LM4 [15], rat cytochromes P-450 c and d [10] and mouse cytochrome P_3-450 [16], the last 4 named being 3-methylcholanthrene-inducible isozymes. It is also absent in mitochondrial cytochrome P-450 (SCC) from the bovine adrenal cortex [17]. Correspondingly, cytochrome P-450 LM4 was a poor substrate for cAMP kinase but a good substrate for protein kinase C prepared from rat brain (unpublished). This raises two interesting questions: whether turnover of cytochromes P-450 generally is regulated by phosphorylation, and whether different kinases regulate turnover of different cytochromes P-450.

A minor aspect of our results relates to the unknown tertiary structure of P-450. The Ser 128 should be located on the surface of the protein near to the heme binding site but not at the binding site of type I substrates – the degree of phosphorylation was not impeded by the presence of benzphetamine, a typical type I substrate, in saturating concentrations (not shown). The charge of the protein surface around Ser 128 appears to be critical for the conformation of cytochrome P-450, as phosphorylation of Ser 128 causes conformational transition of P-450 to cytochrome P-420, which strongly affects the protein environment of the heme and results in drastic spectral changes and a propensity to lose heme [5].

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