Evidence for a crosslink between *c*-heme and a lysine residue in cytochrome P460 of *Nitrosomonas europaea*

David M. Arciero, Alan B. Hooper*

Department of Genetics and Cell Biology, University of Minnesota, 344 Gortner Labs, 1479 Gortner Ave., St. Paul, MN 55108, USA

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Abstract **Cytochrome P460 and hydroxylamine oxidoreductase (HAO) of *Nitrosomonas europaea* catalyze the oxidation of hydroxylamine. Cytochrome P460 contains an unidentified heme-like chromophore whose distinctive spectroscopic properties are similar to those for the P460 heme found in HAO. The heme P460 of HAO has previously been shown by protein chemistry and NMR structural analysis to be a *c*-heme with an additional covalent crosslink between the C2 ring carbon of a tyrosine residue of the polypeptide chain and a *meso* carbon of the porphyrin [Arciero, D.M. et al. (1993) Biochemistry 32, 9370–9378]. The recent determination of the gene sequence for cytochrome P460 [Bergmann, D.J. and Hooper, A.B. (1994) FEBS Lett. 353, 324–326] indicates that the heme in this protein also possesses a *c*-heme binding site and provides the basis for determining whether an HAO-like crosslink exists to the porphyrin.**

Sequence analysis of a purified heme-containing tryptic chromopeptide from cytochrome P460 revealed two predominant amino acid residues per cycle. Two peptides present in the chromopeptide with the sequences NLPTAEXAAXHK and DGTVTVXELVSV. Comparison of the data to the gene sequence for the protein revealed that the gaps in the first peptide (indicated by X's) code for C residues, confirming the prediction of a *c*-heme binding motif. The gap in the sequence in the second peptide at cycle 7 is predicted by the gene sequence to be a K. The results suggest that the lysine residue is crosslinked in some manner to the porphyrin macrocycle, possibly mimicking the tyrosine crosslink found for the heme P460 of HAO. While a common role for the crosslinked residues in HAO and cytochrome P460 is difficult to ascertain due to the dissimilarities in side chain structure, it may be related to the similar pK_a values for lysine and tyrosine.

Key words: Cytochrome P460; Nitrosomonas europaea; Hemeprotein; Heme modification; Proteolytic cleavage

1. Introduction

Nitrosomonas europaea is an autotrophic organism that oxidizes ammonia to nitrite, extracting energy for growth in the process. The critical step appears to be the oxidation of hydroxylamine, an intermediate in the pathway, that provides the electrons and energy. *N. europaea* contains two enzymes capable of oxidizing hydroxylamine. One is the unique octaheme cytochrome, hydroxylamine oxidoreductase (HAO) [1] which apparently confers upon the organism this unique ability to grow on ammonia. The second is cytochrome P460 [2], an enzyme whose metabolic significance has not been shown.

In addition to the functional similarity between the two proteins, spectroscopic evidence suggests that both proteins contain similar heme-like chromophores (referred to as heme P460) essential for catalytic activity. In the case of HAO, which also contains seven *c*-type hemes per subunit [3], the P460 heme has been shown to be a protoheme IX moiety covalently attached to Cys-229 and Cys-232 via thioether linkages as found for typical *c*-hemes, and also to Tyr-467 [4] in a novel C-C linkage between a *meso* carbon of the heme and a ring carbon of the tyrosine [5]. This structure has now been confirmed in the crystal structure of HAO [6]. This is the only known biologically-derived crosslinking of a tyrosine to a heme although a non-biological crosslink can be effected in myoglobin after treatment with hydrogen peroxide [7]. While it is apparent that a modified heme can have significantly different properties relative to a normal *c*-heme, knowing whether this is a universal feature of P460 hemes in general would help evaluate the importance of the tyrosine to heme crosslink found in HAO to the reaction catalyzed by the enzyme.

The gene sequence for cytochrome P460 contains a single *c*-heme binding motif [8]. This has allowed us to address the question of whether a c-heme is attached to the polypeptide at this site and consider whether a crosslink exists in cytochrome P460 and potentially identify the residue involved through protein chemistry. Here we report the results of protein sequencing of proteolytically derived heme-containing polypeptides from cytochrome P460.

2. Materials and methods

Cytochrome P460 was purified as described previously [9]. Trypsin and chymotrypsin were from Worthington Biochemical Corp (Freehold, NJ). Denaturation of cytochrome P460 was carried out in 20 mM KOH anaerobically in the dark at room temperature for 1 hr. Proteolytic digestions were carried out in 0.1 M Tris/acetate buffer, pH 7.8, anaerobically in the dark at room temperature. Peptides were initially fractionated on a Spherogel TSK2000SW HPLC gel filtration column equilibrated with 0.1 M sodium phosphate, pH 7.0 buffer containing 0.1 M sodium sulfate. Subsequent purification was carried out by gradient elution on a Beckman Ultrapore reverse phase HPLC column using a 20 mM ammonium bicarbonate/acetonitrile buffer system. Other details of the HPLC have been reported previously [3]. Chromopeptide fractions eluting between 14.0–14.5 min and 14.5–15.0 min were collected for sequencing. Automated Edman degradation was carried out at the Microchemical Facility at the University of Minnesota on an Applied Biosystems 477A gas phase sequenator. PTH amino acids were analyzed directly on an automated online Applied Biosystems model 120 HPLC system.

3. Results

Lability of the chromophore of cytochrome P460 has hampered all three phases of the process involved in obtaining a

*Corresponding author. Fax: (1) (612) 625-5754.
E-mail: hooper@biosci.cbs.umn.edu

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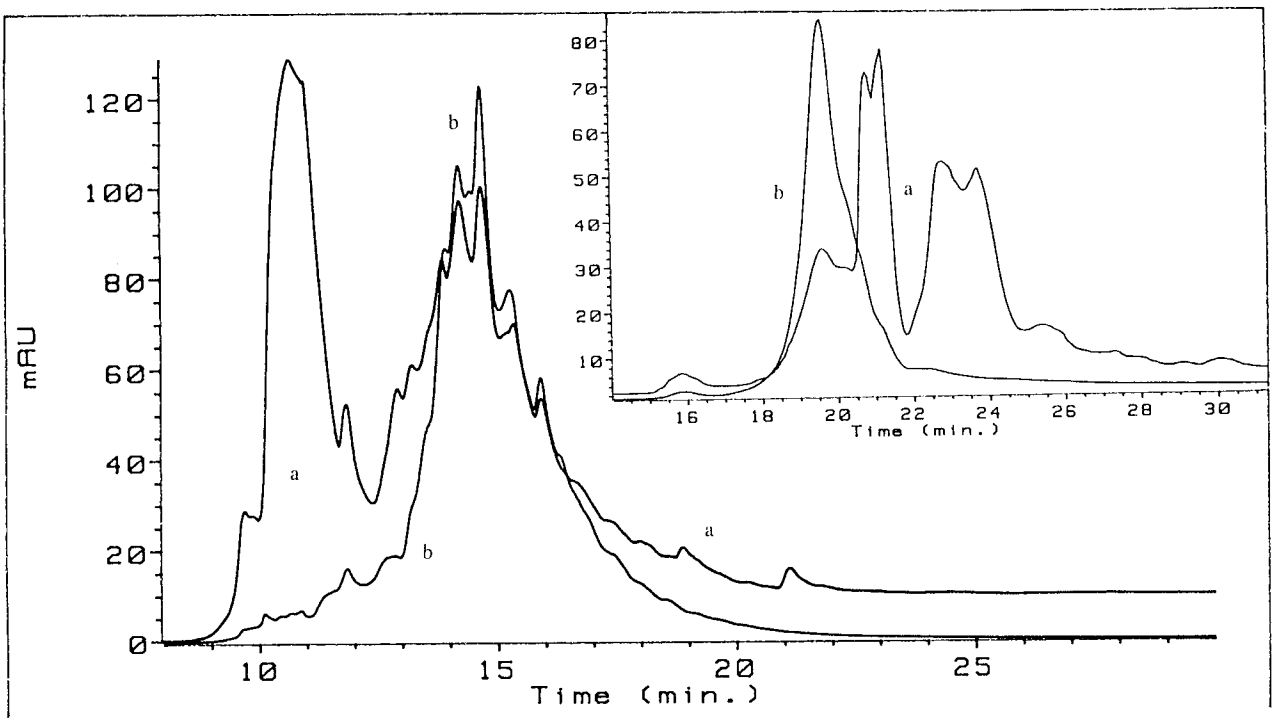

Fig. 1. Reverse phase HPLC chromatogram of polypeptides derived from trypsin digestion of cytochrome P460. Inset shows prior fractionation of tryptic digest on HPLC gel filtration column. Shown is a comparison of traces recorded at 280 (a) and 436 nm (b).

chromopeptide suitable for sequencing. As a result, we have been restricted as to the method of denaturation, choice of protease and length of proteolysis, and conditions for purification of the tryptic chromopeptide. Some of these difficulties have been detailed here. The chromophore was extremely labile especially at acidic values of pH, even under anaerobic conditions; if the protein was denatured anaerobically at pH 2, the Soret absorbance quickly bleached with $t_{1/2}$ of less than an hour and did not return upon neutralization. The chromophore appeared to be much more stable at basic values of pH, although the denaturation of the cytochrome was more difficult. While the optical properties of the chromophore changed dramatically and completely in a short period of time, protein denaturation as detected by changes in absorbance near 310 nm (representing deprotonation of tyrosine residues as they become exposed to the solvent), occurred on a much slower time scale. Even after the absorbance change at 310 nm ceased, the unfolding of the protein was apparently not complete, since upon neutralization of the solution and subsequent proteolytic digestion, only about 50% of the protein was susceptible to proteolysis. To obtain more complete digestion, the protein was therefore subjected to two rounds of base denaturation and proteolysis with only a brief 1 h initial incubation with trypsin. This double denaturation treatment resulted in about 90% proteolysis.

The instability of the chromophore in acid was still a problem after proteolysis. Very rapid degradation occurred if the chromopeptide was subjected to reverse phase chromatography in the trifluoroacetic acid/ acetonitrile buffer system commonly used. The rate of degradation appeared to be appreciably slower, but still evident and problematic, at higher value of pH. The proteolytic digestion mixture was therefore kept at neutral to basic values of pH for isolation of the chromopeptide. While the chromopeptide existed as a heterogeneous mix, dominant chromopeptides existed that could be isolated and sequenced. Initial fractionation of the crude peptide mix was carried out on an HPLC gel filtration column to separate the larger peptides from the bulk of the smaller, non-heme containing peptides. The inset of Fig. 1 shows that the chromopeptide eluted in a single major band. Visible spectra shown in Fig. 2 (top traces) recorded at the leading and following edges, and at and near the maximum show that the chromopeptide fraction was not spectroscopically homogeneous. While the bulk of the material appears homogenous, the material eluting on the wings show a much broader Soret band as well as increased relative absorbance in the 300–400 nm region. Subsequent purification was carried out by gradient elution on a reverse phase C8 column in an aqueous ammonium bicarbonate/ acetonitrile solvent system (Fig. 1). Multiple species with similar retention times can be observed in the

Table 1
Amino acid sequence results for tryptic chromopeptide 14.5–15.0 from cytochrome P460

Cycle	Residue	Yield (pmol)
1	N,D,E	180,115,55
2	L,G,W	196,248,34
3	P,T,V	208,60,57
4	T,V,M	82,143,53
5	A,T,V	176,68,89
6	E,V,G	111,126,100
7	T	25
8	A,E,Q	117,79,32
9	A,L,V	143,75,48
10	V,T	86,14
11	H,S,P	27,15,28
12	K,V,N	56,67,24
13	E,G	56,52
14	N,D,L	23,28,15
15	A,R,N	21,5,17

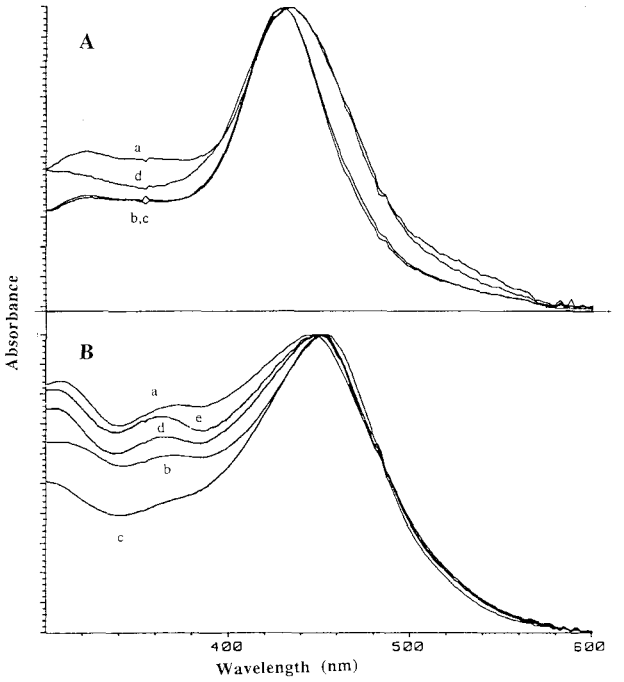

Fig. 2. Optical spectra of chromopeptides derived from trypin digestion of cytochrome P460. Upper traces (A) are spectra recorded at t_R=19.22 (a), 19.59 (b), 19.84 (c), and 20.34 (d) min during the run on the gel filtration column. Lower traces (B) were recorded at t_R = 13.91 (a), 14.22 (b), 14.68 (c), 15.25 (d), and 15.93 (e) min during the run on the reverse phase column.

Abs(436) trace of the chromatogram. These species all have slightly different visible absorption spectra as seen in Fig. 2 (lower traces). There are several major differences between the spectra recorded for the chromopeptides eluting from the two HPLC columns. Chromopeptides eluted from the reverse phase column showed a Soret band that was both broadened and shifted about 20 nm to the red relative to the chromopeptides eluting from the gel filtration column. The chromopeptides on the reverse phase column also show appreciably more absorbance in the 300–400 nm region. Some of these differences may be solvent or buffer related but more likely are due to some degree of degradation. Nevertheless, reasonably clean sequencing results were obtained for various individual chromopeptide species.

The two major chromopeptide peaks, representing more that 60% of the total chromopeptide released from cytochrome P460 with trypsin, were sequenced. Only sequencing results from automated Edman degradation of the sample collected between 14.5 and 15.0 min is shown in Table 1 (the other sample sequenced was the one collected between 14.0 and 14.5 min). Qualitatively, the two sequencing runs were essentially identical. The same two major peptide sequences together with a minor peptide sequence could be identified in both of the sequencing runs. Qualitatively, however, the two sequencing runs were different in the relative amount of the minor peptide present in the samples. While the amount of the minor peptide was about 25% in the run reported here, it was present at about 40% of the most abundant peptide in the other sequencing run. Our inability to purify the chromopeptide free of this minor peptide is largely a reflection of the heterogeneous mixture of chromopeptides resulting from breakdown of the chromophore. Additional attempts to purify the chromopeptide further only led to more heterogeneity. The major peptides had the sequences NLPTAECAACHKENA and DGTVTVKELVSVGDR. The minor peptide present was EWVMVGTQVTPNELN. Analysis of tryptic cleavage sites in the primary sequence of cytochrome P460 obtained from the gene sequence [8] indicate that these three peptide sequences correspond to the tryptic fragments, NLPTAECAACHKENAK, DGTVTVKELVSVGDR, and EWVMVGTQVTPNELNDGK, respectively. Sequencing was only carried out for 15 cycles. Hence, the last three residues of the minor peptide and the last residue of the heme-motif containing peptide can not be accounted for. The second major peptide was sequenced completely based on tryptic fragmentation prediction. In the first 15 cycles, only three of the predicted amino acids could not be accounted for in the sequence analysis and all three were absent in both sequencing runs of the two major chromopeptides analyzed. Two of the amino acids were the cysteine residues (cycles 7 and 10) of the first major polypeptide previously assigned to a *c*-heme binding motif [8] and predicted to be involved in the thioether linkage to the porphyrin. The third was a lysine residue of the second major polypeptide predicted for cycle 7. No other PTH-amino acids (or any unidentified PTH-derivatives) were present in cycle 7 other than those predicted from the sequences of the three polypeptides present.

4. Discussion

The present sequencing results confirm that the heme of cytochrome P460 is attached to the polypeptide chain at a *c*-heme binding site. The evidence is also strong for a covalent crosslink between the tryptic peptide fragment containing the heme moiety and another amino acid residue of the protein. As to the nature of this crosslink, the evidence strongly suggests that a lysine residue is involved and not a tyrosine as in HAO. In addition to the observation that a lysine residue is missing at cycle 7, none of the three tryptic peptides observed are predicted to possess a tyrosine. There remains a remote possibility the crosslink involves an amino acid beyond cycle 15 of the sequencing runs. However, the only residue not accounted for would be the C-terminal K of the peptide containing the *c*-heme binding motif. For the peptide containing the lysine residue believed to be involved in the crosslink, all residues are accounted for based on analysis of tryptic fragmentation patterns.

We have not established that the crosslink is to the porphyrin or that the macrocycle is a porphyrin as has been shown for the P460 heme in HAO. To do so for the P460 heme of HAO was a major undertaking which has not been possible with the P460 heme of cytochrome P460. Besides the extreme lability of the chromophore, cytochrome P460 is only present at about 5% (on a molar basis) relative to the amount of HAO in the organism and more difficult to obtain. It is likely, however, that the crosslink does involve the macrocycle. Resonance Raman spectroscopy has demonstrated that the P460 hemes of both HAO and cytochrome P460 exhibit complex vibrational patterns indicative of macrocycles with lower symmetry than for typical *c*-hemes [10]. Furthermore, the only other potential protein-protein crosslink would have to involve one of the cysteine residues of the *c*-heme binding motif since no other blanks were observed in the amino acid sequence of the heme-containing tryptic peptide.

The finding that a lysine may be involved in the crosslinking in cytochrome P460 instead of a tyrosine as in HAO is somewhat surprising. It seems logical that the crosslinking must confer on both proteins and their heme groups a property not available to *c*-hemes in general. Obviously it is a property that doesn't require one specific crosslink to the porphyrin. The main question is whether the crosslink has a direct or indirect effect on reactivity. Now that the crystal structure of HAO has been described (6), hopefully more detailed analysis can ensue. One possible role may be based on the similar alkaline pK_a values (9.5–10.5) of the two amino acid side chains. The P460 heme of HAO has been shown to have a pH-dependent midpoint potential indicating uptake of a proton accompanying reduction of the iron. This is a property potentially critical to the reaction catalyzed by the enzyme; the concurrent abstraction of both electrons and protons from hydroxylamine. If a similar pH-dependence was found for the heme in cytochrome P460, then the crosslinked residues would be potential sites for accepting protons from substrate. However, for this to be the case the pK_a's would have to be substantially lowered in the crosslinked state. Furthermore, a cytochrome should easily be able to couple proton uptake to electron flow without resorting to crosslinking the ionizable group to the heme group.

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