Secondary structure of NADPH:protochlorophyllide oxidoreductase examined by circular dichroism and prediction methods*

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To study the secondary structure of the enzyme NADPH:protochlorophyllide oxidoreductase (PCOR), a novel method of enzyme isolation was developed. The detergent isotridecyl poly(ethylene glycol) ether (Genapol X-080) selectively solubilizes the enzyme from a prolamellar-body fraction isolated from wheat (*Triticum aestivum* L.). The solubilized fraction was further purified by ion-exchange chromatography. The isolated enzyme was studied by fluorescence spectroscopy at 77 K, and by CD spectroscopy. The fluorescence-emission spectra revealed that the binding properties of the substrate and co-substrate were preserved and that photo-reduction occurred. The CD spectra of PCOR were analysed for the relative amounts of the secondary structures, α-helix, β-sheet, turn and random coil. The secondary-structure composition was estimated to be 33 % α-helix, 19 % β-sheet, 20 % turn and 28 % random coil. These values are in agreement with those predicted by the Predict Heidelberg Deutschland and self-optimized prediction method from alignments methods. The enzyme has some amino acid identity with other NADPH-binding enzymes containing the Rossmann fold. The Rossmann-fold fingerprint motif is localized in the N-terminal region and at the expected positions in the predicted secondary structure. It is suggested that PCOR is anchored to the interfacial region of the membrane by either a β-sheet or an α-helical region containing tryptophan residues. A hydrophobic loop-region could also be involved in membrane anchoring.

INTRODUCTION

The membrane protein, NADPH:protochlorphyllide oxidoreductase (PCOR), catalyses the light-dependent reduction of protochlorophyllide (PChlide) to chlorophyllide (Chlide). PCOR accumulates in the etioplast prolamellar-body (PLB) membranes [1]. In barley and *Arabidopsis*, two different forms of the enzyme have been observed, PCOR A which is expressed only in the dark, and PCOR B which is expressed both in the dark and in the light [2,3]. PCOR forms a ternary complex with NADPH and PChlide. The latter has characteristic absorption maxima at 636 and 650 nm, and a fluorescence maximum at 657 nm [4,5]. The molecular mass of PCOR varies between 33 and 38 kDa [5] and the amino acid sequence, deduced from cDNA clones, is known for several species [6]. A large proportion of the amino acids in PCOR are hydrophobic [7] and the enzyme exhibits amphipathic properties when partitioned with Triton X-114 [8]. However, the hydrophobicity plot shows no hydrophobic segment long enough to form an α-helix which will span the plastid membrane [9].

Many enzymes containing NAD/H, NADP/H or FAD/H as cofactors have been crystallized, and a characteristic dinucleotide-binding domain has been identified. This domain, known as the Rossmann fold, has considerable homology in tertiary structure, and some minor primary amino-acid sequence similarity [10,11]. The Rossmann fold, is formed by an open, parallel, six-stranded β-sheet with α-helices on both sides of the sheet. The domain forms a hydrophobic pocket which binds the dinucleotide. The domain contains a βA-αA-βB motif which has been used as a fingerprint for predicting nucleotide-binding domains [12]. In NAD/H-binding enzymes, the motif is GXGXXG, and in NADP/H-binding enzymes it is modified to GXGXXA [13]. In PCOR, the NADPH-binding domain is thought to be in the N-terminal region, as the first 33 amino acids have sequence similarities with the βA-αA-βB part of the Rossmann fold [14,15]. A conserved arginine residue in this region is also involved in the binding of the 2′-phosphate group of NADPH [15].

Wilks and Timko [16] have suggested that PCOR belongs to a family of short-chain alcohol dehydrogenases, based on protein-sequence alignments. This family has five conserved amino acid residues, two of which are likely to be involved in the hydride-ion transfer from Tyr-275 (Tyr-263 in wheat) and NADPH to PChlide [16].

PCOR is the dominant protein in the PLB membrane [17,18]. This is probably important for the formation of the regularly branched PLB-membrane structure [19]. To understand why the membrane forms this unusual structure, knowledge of the structure of PCOR and its interaction with membrane lipids is of great importance [19]. In this paper we describe the use of CD spectroscopy and prediction models to predict the secondary structure of PCOR. The possible anchoring of PCOR to the PLB membrane is also suggested.

MATERIALS AND METHODS

Isolation of prolamellar bodies

Wheat seedlings (*Triticum aestivum*. L. cv. Kosack Weibulls, Landskrona, Sweden) were grown in a peat and soil mixture

Abbreviations used: Chlide, chlorophyllide; Genapol X-080, isotridecyl poly(ethylene glycol) ether; LDS, lithium dodecyl sulphate; PChlide, protochlorophyllide; PCOR, NADPH:protochlorophyllide oxidoreductase; PHD, Predict Heidelberg Deutschland; PLB, prolamellar body; SOPMA, self-optimized prediction method from alignments.

* This paper is dedicated to our dear friend and colleague, Professor Horst Senger, on the occasion of his 65th birthday.

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containing fertilizer for 6 days at 24 °C. The isolation of the PLB has been described by Widell-Wigge and Selstam [20]. The isolated membranes were stored at −20 °C in 0.5 M sucrose/20 mM Tricine/10 mM Hepes/50 mM KCl buffer (adjusted to pH 7.6 with KOH) containing 0.3 mM NADPH. The isolation of PLB membranes, and the solubilization, purification and spectroscopic measurements of PCOR were performed in dim green light.

Solubilization and specific enzyme activity measurements

To measure the CD spectra of active PCOR in the UV region, a suitable low-absorbing detergent was needed. Additionally, the detergent must preserve PCOR activity. The solubilization experiments were performed according to Hjelmeland and Chrambach [21]. The PLB membranes were solubilized in 0.5 M sucrose/100 mM KCl/0.3 mM NADPH/20 mM tricine (adjusted to pH 7.6 with KOH) containing various types of detergents. The protein concentration was 1 mg/ml and the detergent concentrations were from 0–2.5 % (w/v). After solubilization for 30 min the sample was centrifuged at 150000 ***g*** for 1 h (Ti75 rotor, Beckman) and the supernatant was assayed for enzyme activity [22,23].

The enzyme activity was measured in a double-beam spectrophotometer (Aminco DW-2 UV/Vis, Silver Springs, MA, U.S.A.). The assay media contained the solubilized enzyme fraction, 0.25 mM NADPH and 3.5 nM PChlide. The formation of Chlide was measured after exposure of the sample to light from a photographic flash lamp (Sunpac GX-17) at 20 s intervals.

Purification of PCOR

PLB membrane-proteins (2.5 mg) were solubilized in 1 mM isotridecyl poly(ethylene glycol) ether (Genapol X-080) to a final concentration of 1 mg of protein per ml. The resulting solution was desalted on a PD-10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) which had been equilibrated with 30 ml of elution buffer (0.5 M sucrose/15 mM Tricine/15 mM Mes/0.25 mM NADPH/0.26 mM Genapol X-080, adjusted to pH 5.8 with KOH). After application of the solubilized PLB membranes, the column was eluted with elution buffer. The first 2.5 ml of eluate was discarded and the following 3.5 ml was collected. This desalted fraction was applied (SA-5 sample applicator) to an S–Sepharose Fast Flow cation-exchange column (1.6 × 20 cm) (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with loading buffer (0.4 M sucrose/15 mM Tricine/15 mM Mes/0.25 mM NADPH/0.26 mM Genapol X-080, adjusted to pH 5.8 with KOH). The S–Sepharose column was eluted at 80 ml/h, first with 80 ml of loading buffer and thereafter with 80 ml of 'start' buffer, which was similar to the loading buffer except that the pH was 7.8. Finally, the protein was eluted with an NaCl salt gradient (0 %–100 %) in start buffer over 1.4 h. The elution profile was monitored by absorption spectrophotometry at 280 nm.

The fractions with PCOR activity collected from the cation-exchange column were concentrated in Centriprep-30 concentrators (30 kDa cut-off; Amicon Inc., Beverly, MA, U.S.A.) with centrifugation at 1500 ***g*** and 4 °C (GSA rotor; Beckman) until the sample volume was 1 ml. The samples were desalted in a PD-10 column equilibrated with 30 ml of CD buffer (0.13 mM Genapol X-080/15 mM Tricine, adjusted to pH 7.6 with KOH). The samples were eluted in CD buffer; the first 2.5 ml of eluate was discarded and the following 1.4 ml, containing the PCOR fraction, was collected. The final protein concentration of this fraction was about 0.3 mg/ml. The samples were stored on ice for approx. 1 h before fluorescence and CD were measured.

Aliquots of the concentrated protein fractions were separated by lithium dodecyl sulphate (LDS)/PAGE [24] after solubilization for 10 min at 80 °C in 100 mM Tris (pH 8.6)/160 mM sucrose/1 mM EDTA/1 mM PMSF buffer containing 2 % (w/v) LDS and 0.5 % (w/v) dithiothreitol. The gel was stained for 20 min with 0.2 % (w/v) Coomassie Brilliant Blue R-250 in 20 % methanol and 0.5 % (v/v) acetic acid. Destaining was performed with 30 % methanol. The 38 kDa protein eluted with the salt gradient was excised from the gel and the amino-acid sequence of a segment was determined. The segment was isolated by HPLC [25] after cleavage of the protein with the serine protease from *Staphylococcus aureus* V8.

For quantification of the relative amount of polypeptide isolated by LDS/PAGE, the gels were scanned (Image Master DTS, Pharmacia, Uppsala, Sweden). The gels were stained for 30 min in 0.1 % (w/v) Coomassie Brilliant Blue R-350, 10 % acetic acid and 30 % ethanol (v/v) and destained in a solution of 10 % acetic acid, 30 % ethanol (v/v) in distilled water.

Spectroscopic measurements

CD spectroscopy

The CD spectra were recorded over the range 180–260 nm in a JASCO J-720 spectropolarimeter (JASCO Spectroscopic Co. Ltd., Japan) at room temperature. The optical path length was 0.5 mm and CD buffer was used as a reference. The CD spectrometer was calibrated at 290.5 nm and 192.5 nm using *d*-camphorsulphonic acid.

Fluorescence spectroscopy

The fluorescence spectra of the samples, which were contained in cuvettes cooled in liquid nitrogen, were recorded (SPEX Fluorolog 112, SPEX Ind., NJ, U.S.A.). The excitation wavelength was 440 nm and excitation and emission bandwidths were set to 4.6 nm and 1.8 nm respectively. The emission spectra were corrected. Photo-reduction of PChlide was achieved with three flashes of light from a photographic flash-lamp (Sunpac GX-17).

Secondary-structure estimation

To estimate the proportions of secondary structures (α-helix, β-sheet, turn, random or unordered forms) the reference CD spectra obtained by Yang et al. [26] and Yang [27] were used. The software for analysing the data (SSE-338) and the program used for secondary-structure analysis was developed by JASCO Spectroscopic Co. Ltd., Japan. The program for secondary-structure analysis was based on a simple least-squares method adapted to four secondary structures. The fraction (f_i) of each kind of structure could be constrained by $\Sigma f_i = 1$, or by $f_i > 0$, for each component i. In the present work, only the latter criterion was applied. The secondary-structure estimation used the reference spectra calculated from the CD spectra recorded for myoglobin, lysozyme, ribonuclease A, papain, cytochrome *c*, haemoglobin and α-chymotrypsin. In haemoglobin, the fractions of α-helix, β-sheet, turn and unordered form are given by Manvalan and Johnson [28]. For the remaining proteins, the corresponding fractions are given by Yang et al. [26]. The goodness of fit was judged by inspecting the deviation between the calculated and observed spectra, shown in Figure 4 (line c). The normalized root-mean-square (RMS) residual CD was calculated according to:

$$\mathrm{RMS} = 100 \cdot \sqrt{\frac{\sum_j [\mathrm{CD}_{\mathrm{obs}}(\lambda_j) - \mathrm{CD}_{\mathrm{calc}}(\lambda_j)]^2}{[\mathrm{CD}_{\mathrm{obs}}(\lambda_j)]^2}}$$

where $CD_{obs}(\lambda)$ and $CD_{calc}(\lambda)$ are the observed CD and calculated CD respectively, at the wavelength λ.

The fraction (with error) of secondary structures reported in this work are based on the analysis of three independent preparations of PCOR.

Protein assay

Protein was quantified using the bicinchoninic acid assay according to the manufacturer's instructions (Pierce Chemical Co.) following treatment of a 25 ml sample with 100 ml of 0.1 M KOH at 80 °C for 1 h.

Chemicals

All chemicals were of analytical grade quality. Sucrose (Ultrapure) was from United States Biochemicals (Cleveland, OH, U.S.A.) and Genapol X-080 was from Boehringer-Mannheim, Germany.

Prediction programs

Secondary-structure predictions were made with the Predict Heidelberg Deutschland (PHD) method [29,30] which is available by electronic mail from PredictProtein@EMBL-Heidelberg.DE, and the self-optimized prediction method from alignments (SOPMA), available by electronic mail from deleage@ibcp.fr (subject SOPMA) [31,32]. The sequences analysed by PHD were a multiple-sequence aligning produced by the pileup program in the University of Wisconsin Genetics Computer Group sequence-analysis package (GCG) [33]. The sequences used were *Hordeum vulgare* [34], *Avena sativa* [14], *Arabidopsis thaliana* [9], *Pisum sativum* [35], *Pinus strobus* [36], *Pinus taeda* [36], *Pinus mungo* [37], *T. aestivum* [15] and *Synechocystis* PCC 6803 [6]. For the SOPMA method, the sequence from *T. aestivum* was used.

RESULTS AND DISCUSSION

Solubilization and purification of PCOR

Of several detergents tested for the solubilization of PCOR from PLB membranes, Genapol X-080 yielded the highest specific activity (Figure 1). The increase in specific activity to 180% when PLB membranes were solubilized with 1 mM Genapol X-080 indicated that this detergent preferentially solubilized PCOR relative to other PLB enzymes.

The activity of PCOR solubilized in 1 mM Genapol X-080 was stable on ice for at least 24 h. The detergent tests showed also that, for concentrations between 1 and 5 mM, the total activity of PCOR changed only slightly (Figure 1), indicating that Genapol X-080 solubilized PCOR without destroying the enzyme activity. Most of the other detergents tested were less specific or even inactivated PCOR. The polar-head group of Genapol X-080 is similar to that of Triton X-100 but the hydrophobic chains are different. Genapol X-080 has an aliphatic carbon chain with a branched methyl group (isotridecyl group), while the octylphenol group in Triton X-100 absorbs light in the UV range, making it unsuitable for use in CD spectroscopy. Triton X-100 also solubilized PCOR specifically and, at a concentration of 1 mM, the activity was preserved [38–40] but was stable on ice for only 3 h. Higher concentrations of Triton X-100 inhibited PCOR activity [38,40].

The application of S–Sepharose Fast Flow column chromatography and an NaCl gradient to solubilized PLB membranes resulted in the elution of PCOR at pH 7.8 and 0.2–0.4 M NaCl (Figure 2). The use of a pH gradient was also investigated. At high pH values we found that PCOR was inactivated and degraded. This was surprising, since PCOR has been shown to be more sensitive to high salt concentrations [41] than to high pH values [22]. The salt-inactivation of the enzyme is explained by the electrostatic binding of PChlide to the active site of PCOR [41]. Our success in using ion-exchange chromatography and a salt gradient to elute PCOR could be the use of a desalting procedure on the eluted PCOR fractions, which resulted in reactivation of PCOR. The salt-resistance may also depend on the use of Genapol X-080 instead of Triton X-100.

The eluted proteins were examined by LDS/PAGE. The fraction eluted in 0.2–0.4 M NaCl was shown to contain a polypeptide of 38 kDa. The relative amount of Coomassie Blue

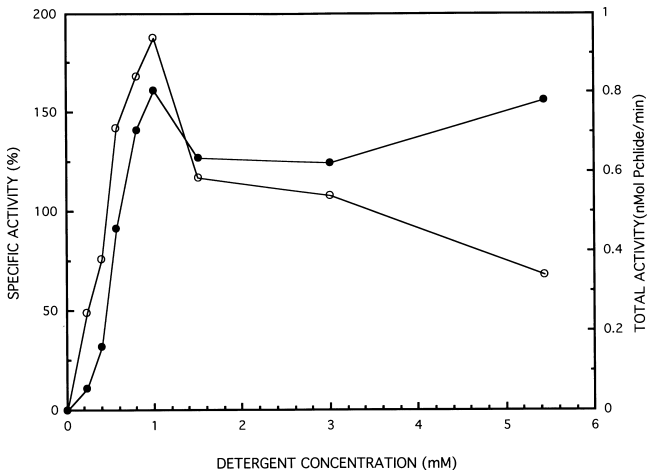

Figure 1 Specific (○) and total activity (●) of PCOR in the supernatant after solubilization and centrifugation of PLB membranes isolated from wheat

The enzyme activity shown is relative to that of the original, untreated PLB membrane fraction. The results shown are based on three independent experiments.

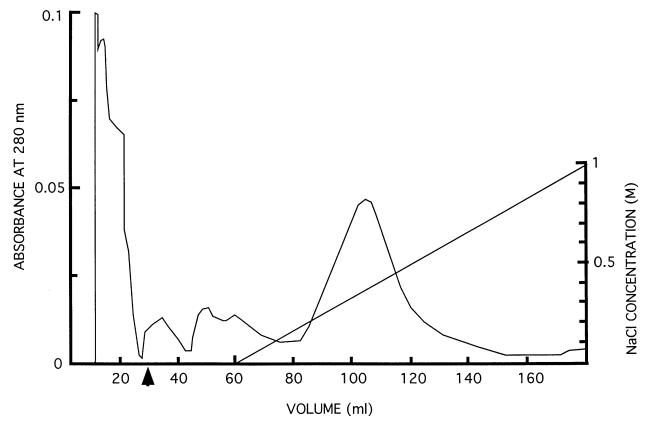

Figure 2 Separation of solubilized PLB membranes by ion-exchange S–Sepharose Fast Flow chromatography

The arrowhead indicates the change of eluting buffer from pH 5.8 to 7.8. The straight line indicates the NaCl concentration. The fractions eluted between 0.2–0.4 M NaCl, which contained PCOR, were pooled. The chromatogram represents the separation of PCOR in the absence of NADPH.

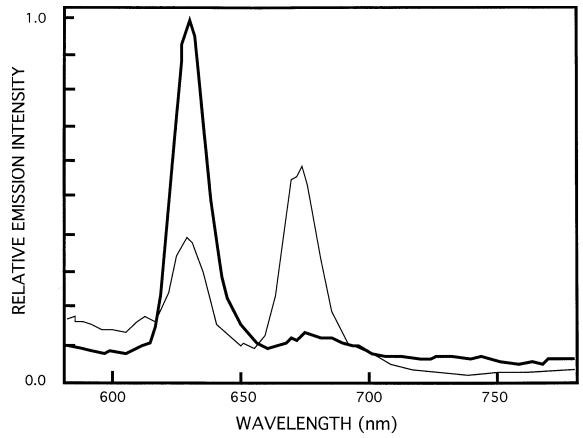

Figure 3 Fluorescence emission spectra of the isolated PCOR before (bold line) and after (thin line) three light flashes

The spectrum of the untreated sample shows a distinct peak at 630 nm. Following illumination, the PChlide peak at 630 nm decreased and a Chlide peak at 675 nm appeared.

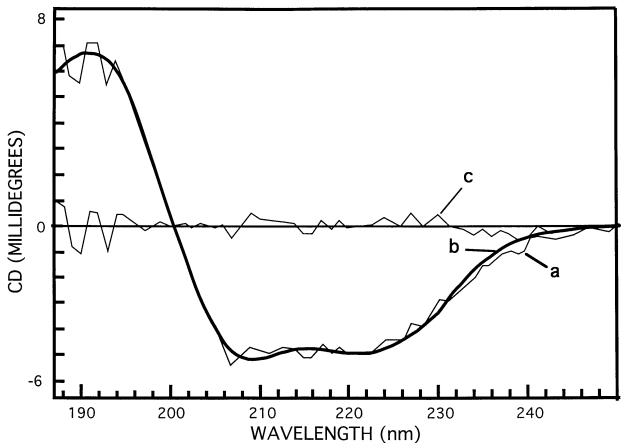

Figure 4 CD spectra of isolated PCOR

The observed spectrum (thin line, a) for isolated PCOR was analysed using a secondary-structure estimation program. The calculated spectrum for this particular experiment (bold line, b) corresponded to 36% α-helix, 19% β-sheet, 17% turn and 28% random coil. An average estimation of 33 ± 3% α-helix, 19 ± 0.2% β-sheet, 20 ± 3% turn and 28 ± 1% random coil structure was obtained for PCOR from three independent experiments. The residual CD spectrum is also shown (thin line, c).

stain found in the 38 kDa band was 84% in the fraction containing the isolated PCOR, while it was 54% in the supernatant of the solubilized PLB fraction. The remaining 16% of the Coomassie Blue stain in the isolated PCOR fraction was found in the diffuse background. The amino acid sequence of a segment of the isolated protein, LSEKLVGLA, was identical with a segment of PCOR at the C-terminal of the protein.

Fluorescence spectra

The crude solubilized PCOR fractions isolated by ion-exchange chromatography were examined by fluorescence spectroscopy at 77 K. In the crude solubilized PCOR fraction, the fluorescence spectra showed two photo-transformable peaks at 630 and 656 nm respectively (results not shown). In the isolated PCOR fraction, the spectrum showed one distinct peak of PChlide at 630 nm (Figure 3). Exposure to three flashes of light resulted in reduction of the 630 nm peak and the appearance of a Chlide peak at 675 nm (Figure 3). This was in contrast with the fluorescence spectra of PChlide observed in isolated PLB membranes, where the emission maxima appear at 633 and 657 nm. The 657 nm peak is considered to be due to the photo-transformable PChlide, which is bound to the active site of the enzyme. *In vivo*, the PChlide fluorescence maximum at 633 nm is thought to be due to free pigment [4,5]. Isolation of PCOR solubilized with Genapol X-080 caused a shift in the fluorescence spectra of the active pigment–protein complex from 657 to 630 nm. The solubilization of the PCOR complex with Triton X-100, CHAPS or SDS also resulted in the formation of complexes with similar blue-shifted absorption and fluorescence maxima [38–40,42], while solubilization with octylglucoside shifted the maxima from 657 to 644 nm [42]. The two spectral properties of the solubilized complexes might be because the octylglycoside complex is an oligomer of PCOR [42], while the Triton X-100 complex is a monomer [39].

Secondary structure from CD spectra

The fractions containing PCOR isolated by ion-exchange chromatography were studied using CD spectroscopy. The spectra obtained were analysed with respect to secondary structure [26]. The wavelength region used was 185–250 nm, which gives more accurate predictions [27]. The CD spectrum of PCOR is shown in Figure 4. The average secondary structure of PCOR obtained from three independent preparations was 33 ± 3% α-helix, 19 ± 0.2% β-sheet, 20 ± 3% turn and 28 ± 1% random coil see (Table 1). The accuracy of secondary-structure predictions from CD spectra is a subject of debate. In the review by Yang et al. [26] the Pearson-product correlation coefficient was calculated for α-helix and β-sheet, and the prediction for α-helix was found to be high (0.87–0.92) while the prediction accuracy for β-sheet was lower but significant (0.25–0.83). The variation in the correlation coefficient, especially for the β-sheet structure, depends on the choice of the reference library [26]. Independent support for our results from CD analysis is given by the computer prediction method which is discussed below.

Secondary structure from computer prediction methods

For comparison and, hopefully, verification of the results obtained by CD, various PCOR amino-acid sequences were aligned and analysed with the PHD and SOPMA secondary-structure prediction programs. The PHD program has a prediction accuracy of about 72% for water-soluble proteins, and both α-helix and β-sheet structures have a good prediction accuracy [29]. Predictions for membrane proteins may be inaccurate [30]. However, PCOR is a hydrophilic membrane protein without long hydrophobic regions [9]. Moreover, the updated PHD method contains an algorithm to identify transmembrane helices. The SOPMA method has a prediction accuracy of 73% [32]. The PHD analysis of PCOR from an alignment of nine sequences from different species predicts a content of 40% α-helix, 18% β-sheet and 42% turns and random coil when the structure is calculated from the sequence with the cleavage site at position 67 [9] (Table 1). With an alignment of different sequences with a declining identity down to 40%, the best possible accuracy in the prediction of the secondary structure is obtained by PHD [30]. With the SOPMA method, the secondary structure of PCOR was 33% α-helix, 13% β-sheet and 54% turns and random coil

Table 1 Secondary structures of PCOR and some other oxidoreductases

The structures of PCOR were obtained by CD spectroscopy and by the PHD and SOPMA prediction methods. The secondary structures of the other oxidoreductases were calculated from crystallized protein structures (PDB, Protein Data Bank, Brookhaven, New York) and analysed by the PHD method. The PCOR sequences used for PHD and SOPMA analyses were the same as those used in Figure 5. The secondary structures are α-helix, β-sheet and others (turns and random coil).

Protein	α-helix (%)	β-sheet (%)	Other (%)	Accession number [Reference] (Source)
PCOR CD	33	19	48	x76532 [15] (EMBL)
PCOR* SOPMA	33	13	54	x76532 [15] (EMBL)
PCOR* PHD	40	18	42	x76532 [15] (EMBL)
Hydroxysteroid DH† PDB	42	23	37	2HSD [48] (PDB)
Hydroxysteroid DH† PHD	35	18	47	P19992 [49] (Swissprot)
Glutathione reductase PDB	42	32	26	1GRB [40] (PDB)
Glutathione reductase PHD	28	26	46	X15772 [50,51] (EMBL) X60373 [52] (Genbank)
Aldose reductase PDB	38	15	47	1ADS [53] (PDB)
Aldose reductase PHD	34	16	50	X15414 [54], X57526 [55] (EMBL)
Trypanothione reductase PDB	37	24	39	1TYP [56] (PDB)
Trypanothione reductase PHD	25	26	49	Z12618 [56] (EMBL)
Alcohol dehydrogenase PDB	33	32	35	1HLD [57] (PDB)
Alcohol dehydrogenase PHD	20	32	48	M64864 [58], X07774 [59] (EMBL)

* Most probable cleavage site for barley and *Arabidopsis* [9].
† Hydroxysteroid DH, 3α(or 20β)-hydroxysteroid dehydrogenase.

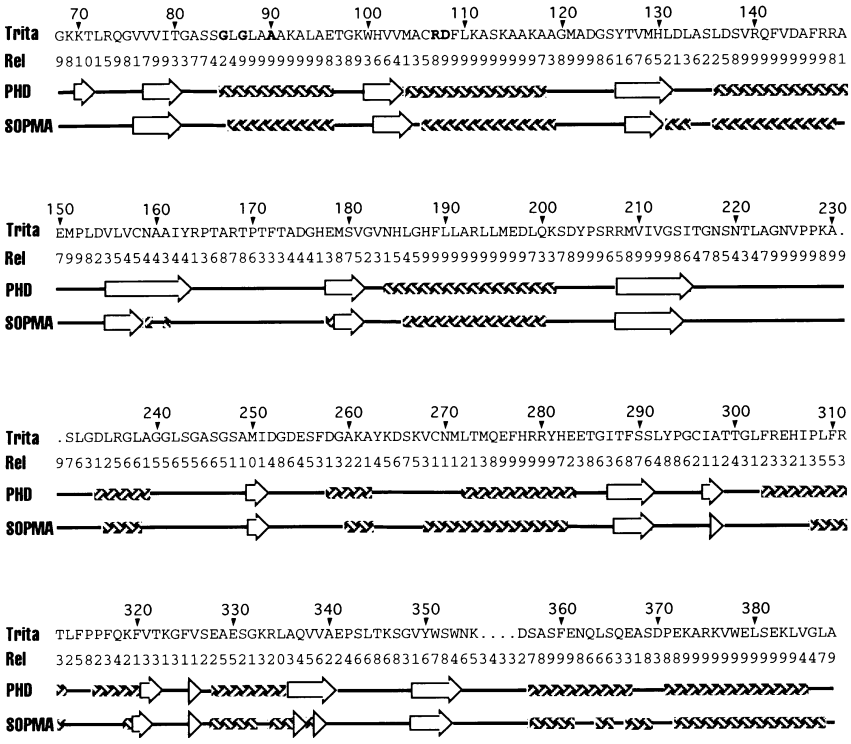

Figure 5 Schematic representation of the PCOR secondary-structure prediction made by the PDH and SOPMA methods

The symbols represent: pleated ribbon, α-helix; arrow, β-sheet; straight line, loop or random coil. Amino acid sequences from *H. vulgare, A. thaliana, A. sativa, P. sativum, P. mungo, P. taeda, P. strobus, Synechcystis* and *T. aestivum* were aligned for the PHD method. The SOPMA prediction was based on the sequence from *T. aestivum*. Abbreviations used: Trita, amino acid sequence from *T. aestivum*; Rel, the reliability index given by the PHDsec method. Bold letters indicate the fingerprint motif in the Rossman fold. Underlined amino acids are the hydrophobic extra loop. The reliability index is given from 0 to 9, where 9 gives an expected overall accuracy of 94.2% and 0 an expected overall accuracy of 72.9% [60].

(Table 1). The predicted values from both PHD and SOPMA methods were in agreement with the values obtained by CD analysis. Not only the proportion of α-helical but also the amount of β-sheet structure found by CD spectroscopy were supported by the prediction. In Table 1 the secondary structures of different oxidoreductases are compared. The secondary structure of PCOR was similar to that of other oxidoreductases, especially hydroxysteroid dehydrogenase and aldolase, even though PCOR is a membrane-bound protein and the other oxidoreductases are water soluble.

The predicted secondary structure illustrated in Figure 5 shows that PCOR is composed of alternating β-sheet and α-helix segments. This alternating arrangement is similar to the secondary structure of the Rossmann fold [11]. However, a barrel structure of parallel β-sheets surrounded by α-helical segments is also possible, similar to that found in NADPH-dependent aldose reductase [43]. It seems most likely that PCOR is of the Rossmann-fold type, since a Rossmann-fold recognition motif is found in the PCOR amino acid sequence. This motif is not found in aldose reductase. The Rossman-fold motif in PCOR is GXGXX(A,T,G)A where A is found in monocotyledons [14,15,34] and T in dicotyledons [9,35] and Gymnosperms [36,37]. Interestingly, the light-dependent PCOR of the cyanobacteria *Synechocystis* [6] has the GXGXXG motif characteristic of an NAD/H-dependent oxidoreductase [13].

In all sequenced PCORs, the fingerprint motif is found close to the N-terminal of the protein. In the Rossmann fold, this motif constitutes the end of the first strand of the β-sheet and marks the start of the following α-helix [12]. In the predicted secondary structure of PCOR (Figure 5), this motif was found in this structural location. Aside from the fingerprint motif, the Rossmann fold has conserved arginine or lysine residues at the N-terminal of the αB-helix, to allow binding of the 2′-phosphate group of NADPH [13]. Four positively charged amino acids are found in this structural position in PCOR, Arg-107, Lys-111, Arg/Lys-114 and Arg/Lys-117 (Figure 5).

When the primary sequence of PCOR is compared with the sequence of other NADPH-binding oxidoreductases, the best sequence homology is seen with NAD/H-binding, short-chain alcohol dehydrogenases, if a long, extra loop of amino acids is inserted between residues 217 and 252 (in wheat, Figure 5) [16]. In the short-chain alcohol dehydrogenases, five amino acid residues are conserved, Gly-87, Asp-154, Ala-160, Tyr-263 and Lys-267 (in wheat). Thus only the second G in the fingerprint motif of the Rossmann fold is conserved, which is the glycine residue interacting with the nicotinamide group. In PCOR from *Synechocystis*, one of these five amino acids, Asp-154, is not conserved. There is also good agreement between the secondary structure of PCOR (see Figure 5) and the short-chain hydroxysteroid dehydrogenase [44].

PCOR–membrane interaction

The part of the PCOR molecule which anchors it to the membrane has not been identified. Neither the hydrophobicity plot nor our prediction of the secondary structure can point to any α-helical structure capable of anchoring the protein in the membrane. On the other hand, other possibilities for membrane anchoring exist, one of which is exemplified by prostaglandin H synthase [45]. Prostaglandin H synthase is a dimer anchored by four amphipathic helices which are oriented parallel to the water–lipid interface of the membrane. The hydrophobic amino acids of each helix face the hydrophobic core of the bilayer, while the hydrophilic residues of the helix reside in the aqueous region. Between these regions, aromatic residues are located at the water–lipid interface [46]. In general, the interfacial regions of membrane proteins are enriched with the aromatic amino acids, tryptophan, tyrosine and phenylalanine [46], where tryptophan can interact with the carbonyl group in the lipid polar-head group [47]. Thus we suggest that PCOR could be anchored to the interface region by an amphipathic segment containing tryptophan. In PCOR, there are four tryptophan residues, one is in the Rossmann-fold region, two in residues 349–353 of the β-sheet structure and one in residues 371–385 of the α-helical structure (Figure 5). In addition, the long 'extra loop' of amino acids (Figure 5) that makes PCOR different from water-soluble short-chain alcohol dehydrogenases, is a hydrophobic region that might also contribute to membrane anchoring.

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