The catechol 2,3-dioxygenase gene of *Rhodococcus rhodochrous* CTM: nucleotide sequence, comparison with isofunctional dioxygenases and evidence for an active-site histidine

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**INTRODUCTION**

The Gram-positive bacterium *Rhodococcus rhodochrous* CTM is able to degrade 2-methylaniline via the meta-cleavage pathway (Fuchs et al., 1991). The enzyme which catalyses the cleavage of the aromatic ring is catechol 2,3-dioxygenase (C230) (EC 1.13.11.2). This enzyme consists of four identical subunits, each of Mr 39000, and contains 1 g atom of Fe²⁺ per subunit (Schreiner et al., 1991). The structural gene for this enzyme is located on a 3.5 kb *BgIII* restriction fragment of plasmid pTC1. We report here the nucleotide sequence of the structural gene for C230. As described by Harayama & Rekik (1989), the extradiol-cleaving enzymes belong to one gene family. All members of this gene family described up to now are produced by different *Pseudomonas* strains (see Table 1).

**METHODS**

**Bacterial strains and vectors.** *Rhodococcus rhodochrous* CTM was isolated from garden soil with 2-methylaniline as sole carbon source (Appel et al., 1984). *Escherichia coli* TG1 (Sambrook et al., 1989) was the recipient strain in transformation and transduction experiments. The vectors were pUC18, M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). The source of DNA for subcloning and nucleotide sequencing was the plasmid pTC1 of *R. rhodochrous* CTM.

**Media and growth conditions.** *R. rhodochrous* CTM was grown at 27 °C in the minimal medium described by Fuchs et al. (1991), with 0.5 % (w/v) sorbitol as carbon source. For plasmid DNA preparations *R. rhodochrous* CTM was cultivated at 27 °C in complex LB medium (Sambrook et al., 1989) with 10 mM threonine. *E. coli* TG1 was grown at 37 °C in M9 minimal medium (Miller, 1972), containing 0.2 % (w/v) glucose as carbon source.

We therefore compared the derived amino acid sequence of C230 from the Gram-positive *R. rhodochrous* CTM with the rest of the gene family, with the aim of obtaining information concerning the active site.

**Keywords:** *Rhodococcus rhodochrous*, catechol 2,3-dioxygenase, aromatic catabolism, extradiol dioxygenases

**Abbreviations:** C230, catechol 2,3-dioxygenase; DEPC, diethylpyrocarbonate; cd, gene encoding catechol 2,3-dioxygenase. The GenBank accession number for the nucleotide sequence reported in this paper is X69504.
carbon source. Recombinant E. coli strains were grown at 37 °C in 2xYT medium or H broth solidified if necessary with 1.5% (w/v) agar (Miller, 1972). Ampicillin at a concentration of 100 µg ml⁻¹ was used for selection of E. coli TG1 containing hybrid plasmids derived from pUC18. For protein purification recombinant E. coli clones were grown overnight at 37 °C in LB medium with ampicillin.

**Enzymes and chemicals.** Restriction endonucleases, T4 DNA ligase, and the T7 Sequencing kit and Deaza T7 Sequencing in 2° recombinant E. coli medium with ampicillin. 3° methylcatechol was from EGA-Chemie; 2,3-dihydroxybiphenyl was from Wako Chemicals; [35S]dATP-aS was from Amersham; 2,3-dihydroxybiphenyl, was from Wako Chemicals; DNA sequencing. Preparative amounts of plasmid DNA from R. rhodochrous were obtained by the method described previously by Schreiner et al. (1991). For analytical purposes recombinant plasmid DNA of E. coli was isolated by the alkaline lysis method (Sambrook et al., 1989). agarose gel electrophoresis, DNA digestion with restriction enzymes, treatment with alkaline phosphatase and ligation were done by standard procedures (Sambrook et al., 1989). DNA fragments were isolated from agarose gels with the GeneClean II kit according to the recommendations of the supplier. Transformation of E. coli with plasmid DNA was performed by the CaCl₂ procedure (Maneli & Higa, 1970).

**Recombinant plasmid construction.** Plasmid DNA from R. rhodochrous CTM was digested with the appropriate restriction endonucleases and size-fractionated by agarose gel electrophoresis. After extraction from the agarose gel the desired restriction fragments were ligated into the linearized vector pUC18 (Fig. 1). pUC18 was digested with either the equivalent enzyme or an enzyme producing compatible ends (e.g., BamHI/BglII) and then treated with shrimp alkaline phosphatase. Ligation was carried out with 2.5 µg insert DNA, 0.5 µg vector DNA and 1 unit T4 DNA ligase. After transformation of competent E. coli TG1, ampicillin-resistant white colonies on 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside plates were isolated by the alkaline lysis method (Sambrook & Russell, 1989). DNA fragments were isolated from agarose gels with the GeneClean II kit according to the recommendations of the supplier. Transformation of E. coli with plasmid DNA was performed by the CaCl₂ procedure (Maneli & Higa, 1970).

**DNA sequencing.** The 3.5 kb BglII fragment of plasmid pTC1 from R. rhodochrous CTM was isolated for subcloning into M13mp18 and M13mp19. Fragments of the 3.5 kb BglII fragment generated by digestion with different restriction enzymes were introduced into vector M13 (Fig. 1). Transformation of E. coli with M13 phage DNA, preparation of single-stranded DNA from selected phage plaques and dideoxy sequencing reactions were performed according to the instruction manual provided by Pharmacia with the sequencing kit for the use of [35S]dATP-aS. All stretches of DNA were sequenced in both directions with the dideoxynucleotide sequencing method of Sanger et al. (1977). Sequence ladders were resolved on gels containing 6% (w/v) polyacrylamide. The nucleotide sequences were analysed with the GENMON program (Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG).

**Purification of C230 from E. coli clones.** Frozen cells (1 g wet weight per ml buffer) of E. coli clones were suspended in 50 mM potassium phosphate buffer, pH 7.5, and disrupted for 20 min by sonification with a Branson Sonifier 450. After centrifugation (48000 g) the cell-free extract was heated to 45 °C for 5 min and centrifuged again. All following purification steps were done at 20 °C. The supernatant was applied onto a Q Sepharose Fast Flow column (14 x 2.3 cm), previously equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Enzyme was eluted with a 0-0.8 M linear KCl gradient in 50 mM potassium phosphate buffer, pH 7.5. Fractions containing active C230 were combined and brought to 20% saturation with ammonium sulphate. Further purification was obtained by hydrophobic interaction chromatography on Butyl-Sepharose CL-4B (7.5 x 3 cm). The enzyme was eluted with a linear ammonium sulphate gradient (20-0% saturation). Fractions with C230 activity were combined and desalted by dialysis with 50 mM potassium phosphate buffer, pH 7.5, and ultrafiltration with a PM 10 membrane (Amicon). The pooled fractions were then again applied onto the Q Sepharose Fast Flow column and eluted with a 0-0.8 M linear KCl gradient as described above. The active fractions were collected, concentrated to 3 ml by ultrafiltration and stored at 4 °C. This enzyme solution was used for modification with diethylpyrocarbonate.

**Reactivation of C230.** The incubation mixture contained 50 mM Tris/acetate buffer, pH 7.5, and 1-4 mg of enzyme in a final volume of 1 ml. The following additions to the incubation mixture were made as indicated: 2 mM ferrous ammonium sulphate (Fe²⁺), 5 mM ascorbic acid or 2 mM cysteine. HCl. Incubation was carried out at 24 °C. An aliquot of the mixture (0.1 ml) was used for the spectrophotometric assay of enzyme activity. Reactivation of C230 after every step of purification was done in the same way, using Fe²⁺ and ascorbic acid.

**Partial purification of C230.** To check for the presence of two C230s in E. coli clones, fractions from the first Q Sepharose Fast Flow column described above were examined for enzyme activity with 2,3-dihydroxybiphenyl and 3-methylcatechol as substrates. Active fractions were combined and desalted by dialysis with 50 mM potassium phosphate buffer, pH 7.5, and ultrafiltration with an Amicon PM 10 membrane. The sample was subjected to a second anion-exchange chromatography using a MonoQ HR 5/5 column connected with an FPLC system and eluted with a 0-0.8 M linear KCl gradient in 50 mM potassium phosphate buffer, pH 7.5. Fractions were analysed for enzyme activity with both substrates.

Frozen cells (0.5 g wet weight per ml buffer) of R. rhodochrous CTM were suspended in 50 mM potassium phosphate buffer, pH 7.5, and disrupted by three passages through a French press at 20000 p.s.i. (about 138 MPa). After centrifugation (48000 g) the cell-free extract was analysed as described above for extracts of E. coli clones.

**Enzyme assay.** C230 activity was determined at 25 °C. The standard assay volume of 1 ml contained 50 mM potassium phosphate buffer, pH 7.5, and 100 µl 5 mM 2,3-dihydroxybiphenyl dissolved in acetone or 300 µl 5 mM 3-methylcatechol in water. The reaction was started by adding an appropriate amount of enzyme. The formation of 2-hydroxy-6-oxo-6-phenylhexadienoic acid, the meta-cleavage product of 2,3-dihydroxybiphenyl, was followed spectrophotometrically at the absorption maximum of 432 nm and quantified using the absorption coefficient reported by Catelani & Colombi (1974). The amount of 2-hydroxy-6-oxohepta-2,4-dienoic acid formed from 3-methylcatechol was estimated as described by Rast et al. (1980). One unit (U) of activity was defined as the amount of enzyme which cleaves 1 µmol 2,3-dihydroxybiphenyl or 3-methylcatechol min⁻¹. Specific activity was based on protein determined by the method of Bradford (1976) with bovine serum albumin as the standard.

**SDS-polycrylamide gel electrophoresis.** SDS-PAGE was carried out according to the method of Laemmli (1970) using a
Catechol 2,3-dioxygenase gene of *Rhodococcus*

Fig. 1. Restriction map of the *R. rhodochrous* plasmid DNA region containing cdo. The bold line below cdo indicates the sequenced region shown in Fig. 3, which was established by sequencing the M13 subclones shown above the map. In the plasmid constructs below the map, the open arrowheads denote the position and direction of the lac promoter of pUC18. Expression of C230 in clones harbouring the corresponding plasmid is shown in parenthesis. Abbreviations: A, Apal; B, BamHI; Bg, BgIII; (Bg), BgIII site lost after cloning; E, EcoRI; Eo, EcoRV; H, HindIII; M, MluI; Nr, NruI; P, PvuI; S, SacI; Sm, SmaI; Ss, SstI; X, Xhol.

RESULTS AND DISCUSSION

Detection of C230 activity after partial purification

Schreiner *et al.* (1991) described the expression of two C230s from the 3.5 kb *Bgl*II fragment (pSC3501, pSC3502; Fig. 1). However, using ion-exchange chromatography (FPLC) as described by them, only one C230 could be found (Fig. 2). In cell-free extracts of *R. rhodochrous* CTM partially purified by ion-exchange chromatography, again only one C230 could be detected. This enzyme accepted 3-methylcatechol as well as 2,3-

dihydroxybiphenyl.
The nucleotide sequence of the 2157 bp EcoRI-BgIII fragment from the R. rhodochrous plasmid pTC1 containing the cdo gene. The ORF commences at base 496 and terminates at 1458. The predicted amino acid sequence is indicated above the DNA sequence. Underlined amino acid sequences are identical with those determined by Edman degradation. A potential ribosome binding site (marked as SD) and an E. coli promoter-like sequence (marked as -10 and -35) upstream of the ORF are indicated. The arrows designate inverted repeat sequences downstream of the termination codon (indicated by asterisks).

Subcloning

In order to elucidate the contradiction between our results and those of Schreiner et al. (1991), we repeated a subcloning experiment already described by them. The 1.2 kb BgAI-SmaI, the 1.15 kb SmaI and the 2.35 kb BglI-SmaI fragments (pSC1200, pSC1150, pSC2350; Fig. 1) were cloned in pUC18. In contrast to the results of Schreiner et al. (1991), only insertion of the 2.35 kb BglI-SmaI fragment into pUC18 led to expression of C230. The recombinant plasmids pSC1200 and pSC1150 yielded no positive clones. This suggests that only one gene for C230 was present in the region between the first BgAI and the second SmaI restriction site (Fig. 1), and that the first SmaI restriction site was situated inside the structural gene.

Clones with the 3.5 kb BgAI or the 1.7 kb EcoRI fragment constitutively expressed a single C230. The enzyme was expressed in both orientations with regard to the lac promoter of pUC18 (Fig. 1). This means that the structural gene.

Dihydroxybiphenyl and was thus identified as C230 II described by Schreiner et al. (1991). From these findings we conclude that C230 I observed by them was probably an artefact.
Table 1. Extradiol dioxygenases belonging to the same gene family

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Bacterium</th>
<th>Plasmid</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdo</td>
<td>Catechol 2,3-dioxygenase</td>
<td><em>Rhodococcus rhodochrous</em> CTM</td>
<td>pTC1</td>
<td>Schreiner et al. (1991)</td>
</tr>
<tr>
<td>dmpB</td>
<td>Catechol 2,3-dioxygenase</td>
<td><em>Pseudomonas</em> CF600</td>
<td>pV1150</td>
<td>Bartillon &amp; Shingler (1989)</td>
</tr>
<tr>
<td>nahH</td>
<td>Catechol 2,3-dioxygenase</td>
<td><em>Pseudomonas putida</em></td>
<td>NAH7</td>
<td>Ghosal et al. (1987)</td>
</tr>
<tr>
<td>xylE1</td>
<td>Catechol 2,3-dioxygenase</td>
<td><em>Pseudomonas putida</em> rev-2</td>
<td>TOL plasmid pWW0</td>
<td>Harayama et al. (1987)</td>
</tr>
<tr>
<td>xylE2</td>
<td>Catechol 2,3-dioxygenase</td>
<td><em>Pseudomonas putida</em> HS1</td>
<td>TOL plasmid pDK1</td>
<td>Nakai et al. (1983)</td>
</tr>
<tr>
<td>indE</td>
<td>3-Methylcatechol 2,3-dioxygenase</td>
<td><em>Pseudomonas putida</em> F1</td>
<td></td>
<td>Benjamin et al. (1991)</td>
</tr>
<tr>
<td>hphC1</td>
<td>2,3-Dihydroxybiphenyl dioxygenase</td>
<td><em>Pseudomonas putida</em> F1</td>
<td></td>
<td>Zukowski et al. (1989)</td>
</tr>
<tr>
<td>hphC2</td>
<td>2,3-Dihydroxybiphenyl dioxygenase</td>
<td><em>Pseudomonas putida</em> F1</td>
<td></td>
<td>Zylstra &amp; Gibson (1989)</td>
</tr>
<tr>
<td>hphC3</td>
<td>2,3-Dihydroxybiphenyl dioxygenase</td>
<td><em>Pseudomonas putida</em> F1</td>
<td></td>
<td>Furukawa et al. (1987)</td>
</tr>
<tr>
<td>nahC</td>
<td>1,2-Dihydroxynaphthalene dioxygenase</td>
<td><em>Pseudomonas putida</em> F1</td>
<td>NAH7</td>
<td>Taia et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kimbara et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Harayama &amp; Rekik (1989)</td>
</tr>
</tbody>
</table>

Inverted repeat sequences, which may act as transcription terminators (Rosenberg & Court, 1979), were found in the 3' flanking region downstream from the termination codon.

The 60.7 mol % G + C content of the *cdo* gene is similar to the 63–72 mol % G + C content of the genus *Rhodococcus* (Goodfellow, 1986). Thus the chromosomal G + C content of the host appears to be maintained even at the level of a structural gene carried on a plasmid. As would be predicted from the high G + C content of the *cdo* gene, its codon usage is highly biased (75 %) in favour of G or C in the wobble base.

Similarity of C23O with other meta-cleavage enzymes

The comparison of amino acid sequences of related enzymes is of particular interest, because conserved amino acid residues may be important for enzyme function. Therefore the derived amino acid sequence of C23O was compared to that of the various extradiol dioxygenases reported already. As described by Harayama & Rekik (1989) some of these enzymes are members of the same gene family. Since 1989 the sequences of another four extradiol-cleaving enzymes have been published, which are similar to those of the above-mentioned family. Together with that of the C23O of *R. rhodochrous* CTM, it is now possible to compare the amino acid sequences of 10 extradiol dioxygenases (Table 1).

In Fig. 4 these amino acid sequences are shown optimally aligned by introducing gaps to maximize identities using the GEnMon program. C23O of *R. rhodochrous* CTM is about 30 % homologous to the derived amino acid sequences of NAHH, *XYLE1*, *XYLE2* and DMPB. The remaining sequences (TODE, BPHC1, BPHC2, BPHC3, NAHC; for references see Table 1) could be aligned with that of C23O with about 20 % identical residues. The
The gene family of extradiol dioxygenases can be divided into two subfamilies comprising dioxygenases for monocyclic and bicyclic compounds, respectively. Members of each subfamily exhibit greater homology among themselves than to members of the other subfamily. Besides these extradiol-cleaving enzymes belonging to the upper-case letters mean 100% conservation in all sequences examined and the lower-case letters conservation in more than 50% of the sequences. The numbering system refers only to the amino acid arrangement in this figure and not to any of the sequences. The sequences were obtained from the sources indicated in Table 1.

Table 2. Degree of homology between C230 and the gene family members

<table>
<thead>
<tr>
<th>Comparison between C230 and:</th>
<th>Identical Degree of amino acids (%)</th>
<th>Identical Degree of nucleotides homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAHH</td>
<td>94/307</td>
<td>31</td>
</tr>
<tr>
<td>XYLE1</td>
<td>95/307</td>
<td>31</td>
</tr>
<tr>
<td>XYLE2</td>
<td>93/307</td>
<td>30</td>
</tr>
<tr>
<td>DMPB</td>
<td>94/307</td>
<td>31</td>
</tr>
<tr>
<td>TODE</td>
<td>56/290</td>
<td>19</td>
</tr>
<tr>
<td>BPHC1</td>
<td>50/302</td>
<td>17</td>
</tr>
<tr>
<td>BPHC2</td>
<td>53/299</td>
<td>18</td>
</tr>
<tr>
<td>BPHC3</td>
<td>50/292</td>
<td>17</td>
</tr>
<tr>
<td>NAHC</td>
<td>62/302</td>
<td>21</td>
</tr>
</tbody>
</table>

degrees of homology based on comparison of the amino acid sequences and of the nucleotide sequences are summarized in Table 2. In all cases the homology between the nucleotide sequences is higher than that of the amino acid sequences. This may indicate a common genetic origin of all these enzymes. However, it is remarkable that the extradiol dioxygenase of a Gram-positive organism has such high similarities to isofunctional enzymes from Gram-negative Pseudomonas species.

The gene family of extradiol dioxygenases can be divided into two subfamilies comprising dioxygenases for monocyclic and bicyclic compounds, respectively. The members of each subfamily exhibit greater homology among one another than to members of the other subfamily. Besides these extradiol-cleaving enzymes belonging to the upper-case letters mean 100% conservation in all sequences examined and the lower-case letters conservation in more than 50% of the sequences. The numbering system refers only to the amino acid arrangement in this figure and not to any of the sequences. The sequences were obtained from the sources indicated in Table 1.
mentioned gene family members. However, their amino acid sequences did not match to the alignment of the gene family of dioxygenases. The same applies to the derived amino acid sequence of the homoprotocatechuate 2,3-dioxygenase from *E. coli* C (Roper & Cooper, 1990). The extent of similarity of this enzyme with any of the aromatic ring-fission dioxygenases (extradiol and intra-diol) varied between 8% and 14%, indicating that there was little or no relationship.

These three extradiol dioxygenases do not belong to the gene family, but they consist of identical subunits of Mr about 30000-40000, like the members of the extradiol dioxygenase gene family. In contrast, the protocatechuate 4,5-dioxygenase from *Pseudomonas putidomalis* consists of an equal number of two different subunits, α and β, of Mr 18000 and 34000, respectively (Noda et al., 1990). The amino acid sequences of these subunits are not related to those of the main C23O family, but the β-subunit of protocatechuate 4,5-dioxygenase exhibits sequence similarity to that of the C23O from *Alcaligenes eutrophus* (Harayama et al., 1992).

Thus it can be suggested that some of the meta-cleavage dioxygenases originated from a common ancestor. However, besides this gene family, other iso-functional dioxygenases must have evolved separately. This suggestion, based on comparison of amino acid sequences, is supported by the finding that each naturally occurring TOL plasmid examined by Keil et al. (1985) and Chatfield & Williams (1986) appeared to carry either two homologous or two nonhomologous genes for C23O. Furthermore, Olson et al. (1992) described a 3,4-dihydroxyphenylacetate 2,3-dioxygenase in soil *Artrobacter* strains not having any immunological similarity to several C23Os of *Bacillus* and *Pseudomonas* strains. This unique type of extradiol-cleaving dioxygenase seemed to be confined to *Artrobacter* soil bacteria.

**Conserved amino acid residues**

Residues that are important for enzyme activity may be found among the conserved amino acids. Altogether 18 amino acid residues have been conserved among the compared extradiol dioxygenase sequences (Fig. 4). It is noteworthy that five glycines and two prolines are among these. The contribution of glycyl and prolyl residues is the primary sequences suggests that the tertiary structures will prove to be highly conserved among the extradiol dioxygenases.

The active site of C23O contains a mononuclear high-spin Fe^{2+} centre that has been implicated in the binding and catalytic activation of molecular oxygen (Mabrouk et al., 1991). It has been suggested that histidine and/or tyrosine may be the iron-binding residues in C23O (Tatsuno et al., 1980), as is the case for protocatechuate 3,4-dioxygenase, an intradiol-cleaving enzyme (Ohlendorf et al., 1988). Therefore the high conservation of four histidines and one tyrosine in all 10 enzymes compared is very interesting. Williams et al. (1990) constructed hybrid *xy/E* genes to define the region important for enzyme activity.

Their results suggested that the main active-site amino acids were located in the C-terminal region. Three of the four conserved histidines and the conserved tyrosine are situated in the C-terminal region, indicating their putative importance for enzyme activity.

Taira et al. (1988) suggested that cysteine might be functionally important due to the formation of disulphide linkages between the subunits of extradiol dioxygenases. However, this cannot be the case with C23O, as this enzyme does not contain any cysteine.

**Purification of C23O from *E. coli* clones**

*E. coli* clones harbouring the recombinant plasmid pSC1701 (Fig. 1) were used for enzyme purification, because these clones produced the highest amount of C23O per g cells. The purification procedure allowing the isolation of 2-6 mg homogeneous C23O (Fig. 5) per g cells (wet weight) is summarized in Table 3.

C23O from *R. rhodochrous*, like that from other bacteria, is easily inactivated during the purification procedure (see Table 3). This inactivation appears to be due to oxidation of Fe^{2+} to Fe^{3+}. The enzyme, thus inactivated, can be partially reactivated by incubation with ferrous ion and a reducing agent (Nozaki et al., 1968). In this way an
Table 3. Purification of C230 from R. rhodochrous CTM, cloned in E. coli TGI

Values in parentheses: purification with reactivation of C230 after every step.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity [mU (mg protein)⁻¹]</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract*</td>
<td>26 (32)</td>
<td>1482 (1776)</td>
<td>258 (288)</td>
<td>174 (162)</td>
<td>1·0 (1·0)</td>
</tr>
<tr>
<td>Heat precipitation</td>
<td>23 (27)</td>
<td>1166 (1161)</td>
<td>253 (268)</td>
<td>217 (231)</td>
<td>1·3 (1·4)</td>
</tr>
<tr>
<td>Q Sepharose Fast Flow</td>
<td>49 (48)</td>
<td>403 (122)</td>
<td>56 (123)</td>
<td>138 (1003)</td>
<td>0·8 (6·2)</td>
</tr>
<tr>
<td>Butyl-Sepharose CL-4B</td>
<td>165 (139)</td>
<td>147 (53)</td>
<td>14 (54)</td>
<td>97 (1024)</td>
<td>0·6 (6·3)</td>
</tr>
<tr>
<td>2nd Q Sepharose Fast Flow</td>
<td>46 (45)</td>
<td>49 (33)</td>
<td>1·1 (32)</td>
<td>22 (972)</td>
<td>0·1 (6·0)</td>
</tr>
</tbody>
</table>

*Prepared from 16 g cells (wet wt).

Table 4. Activation and reactivation of C230

<table>
<thead>
<tr>
<th>Enzyme preparation and addition</th>
<th>Specific activity (mU mg⁻¹)</th>
<th>Activation (-fold)</th>
</tr>
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<tbody>
<tr>
<td>Untreated*</td>
<td>29</td>
<td>1·0</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>162</td>
<td>5·6</td>
</tr>
<tr>
<td>Ascorbate + Fe²⁺</td>
<td>179</td>
<td>6·2</td>
</tr>
<tr>
<td>Cysteine + Fe²⁺</td>
<td>205</td>
<td>7·1</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>170</td>
<td>5·9</td>
</tr>
<tr>
<td>Inactivated with DEPC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>178</td>
<td>6·1</td>
</tr>
</tbody>
</table>

*Untreated enzyme preparation means enzyme partially inactivated by the purification procedure.

enzyme activity six to seven times higher than that found after the purification procedure could be obtained (Table 4). Even if reactivation was carried out after every step of purification, the C230 could not be isolated in a fully reactivated form (see Table 3, values in parentheses).

Modification of histidine by diethylpyrocarbonate

To elucidate the role of the conserved histidine residues, we studied the effect of chemical modification by DEPC on the activity of C230. For this purpose, we used C230 purified without reactivation after every step.

Inactivation of an enzyme by DEPC may be correlated with the modification of histidyl residues if hydroxylamine reactivates the enzyme. C230 was inactivated by DEPC in a time-dependent and concentration-dependent manner. The time-dependent formation of N-carbethoxyhistidine can be monitored by the increase in absorption between 230 and 250 nm (Miles, 1977). The absorption spectra at various times after addition of 25 μl DEPC are shown in Fig. 6(a). In Fig. 6(b) the calculated number of N-carbethoxyhistidines formed after addition of different amounts of DEPC is plotted against time. Parallel to the modification, concentration-dependent inactivation of C230 could be measured (Fig. 6c). The enzyme was totally inactivated within 10 min after addition of 25 μl DEPC (7·5 μmol) and within 45 min after addition of 10 μl DEPC (3 μmol). Addition of 5 μl DEPC (1·5 μmol) led to a decrease of enzyme activity to 10% residual activity within 60 min.

C230 could be reactivated by hydroxylamine. Two hours after addition of hydroxylamine the enzyme was fully active again. (The term 'fully active' means here the maximal activity obtainable if the purified untreated enzyme was reactivated with Fe²⁺ and reducing agents.) Addition of hydroxylamine to either DEPC-inactivated or untreated C230 led to an activation up to the same extent within 2 h. Longer incubation with hydroxylamine resulted in a total and irreversible loss of enzyme activity.

Although DEPC reacts preferentially with histidyl groups, under certain conditions it may also react with cysteine, lysine and tyrosine (Miles, 1977). In contrast to the reaction with histidine and tyrosine, the reaction with cysteine and lysine is not reversible by addition of hydroxylamine. Furthermore, the formation of modified tyrosyl residues would be indicated by a decrease of absorption at 278 nm, but only an increase of absorption between 230 and 250 nm due to N-carbethoxyhistidine was observed (Fig. 6a). Thus the inactivation of C230 by incubation with DEPC is exclusively due to the modification of histidyl residues.

Our results imply that at least one of the four strictly conserved histidines is indeed important for enzyme activity. This finding is supported by a site-specific mutagenesis experiment reported by Taira et al. (1992). They exchanged the strictly conserved His²³⁴ of TODE, BPHC1, BPHC2 and BPHC3 (for references see Table 1) and the nonconserved His²³³ and His²³⁸ of BPHCl with alanine (for the numbering of histidines see Fig. 4). The exchange of the conserved His²³⁴ completely abolished enzyme activity. On the other hand, mutagenesis of the nonconserved histidines had no effect on enzyme activity. They concluded His²³⁴ to be part of the binding site for Fe²⁺.

Further investigations, in particular site-directed mutagenesis experiments, will show whether all four conserved
histidines are essential for enzyme activity. The possible participation of the strictly conserved tyrosine (Tyr^275\footnote{Fig. 4}) in the binding of Fe^{3+} also merits further study.

**ACKNOWLEDGEMENTS**

We are grateful to K. Kapassakalis for skilful fermentation.

This work was supported by a Heisenberg grant from the Deutsche Forschungsgemeinschaft (K.-H. van Pée), by the Bundesministerium für Forschung und Technologie (FRG) and by the Fonds der Chemischen Industrie.

**REFERENCES**


Received 15 December 1992; revised 12 August 1993; accepted 15 September 1993.