Generation of novel functional metalloproteins via hybrids of cytochrome c and peroxidase

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The continued interest in protein engineering has led to intense efforts in developing novel stable enzymes, which could not only give boost to industrial and biomedical applications, but also enhance our understanding of the structure–function relationships of proteins. We present here the generation of three hybrid proteins of cytochrome c (cyt c) and peroxidase via structure-based rational mutagenesis of cyt c. Several residues (positions 67, 70, 71 and 80) in the distal heme region of cyt c were mutated to the highly conserved amino acids in the heme pocket of peroxidases. The multiple mutants were found to exhibit high peroxidase activity and conserve the impressive stability of cyt c. We expect that this strategy could be extended to other cases of metalloprotein engineering, and lead to the development of stable and active biocatalysts for industrial uses. Besides, this study also provides insight into the structure–function relationships of hemoproteins.

Keywords: cytochrome c/peroxidase/protein engineering

Introduction

One of the most exciting topics in protein engineering comes from the engineering of metalloproteins (Finkelstein, 2009; Lu et al., 2009). The rich coordination chemistry of metal ions or metal cofactors, as well as the wealth of functional diversity, makes metalloproteins highly promising candidates for protein engineering.

Cytochrome c (cyt c), a small hemoprotein, is unique among metalloproteins in that its metal cofactor heme is covalently bound to protein peptide via two thioether linkages (Bertini et al., 2006). Such covalent linkages promote the stability of cyt c, making it much less likely to lose its metal cofactor than other metalloproteins. Cyt c could perform some reactions in extreme conditions (very acidic or organic solvents, etc.). Thus, it could serve as an excellent structural framework in the engineering of stable enzymes for industrial uses. Although cyt c engineering has attracted considerable attention, e.g. for use as biosensors (Wallace and Corthesy, 1986; Sorrell and Martin, 1989; Lett and Guillemette, 2002; Baddam and Bowler, 2005b), there has been relatively little focus on the engineering of cyt c for biocatalytic applications (Ying et al., 2011). It is probably because the heme center of cyt c is compactly surrounded by the distal polypeptide chains and also coordinated with its sixth axial ligand Met80, which excludes heme pocket from the protein, and thus makes it difficult to introduce catalytic activity into cyt c. Interestingly, it was found that cyt c could switch to a ‘pro-apoptotic’ conformation with increased peroxidase activity and can catalyze cardiolipin peroxidation, an event which leads to mitochondria outer membrane permeabilization, causing death of the cell (Kagan et al., 2005; Ow et al., 2008; Ying et al., 2009a). These findings therefore provide a rational basis for the development of stable and peroxidase-catalytic enzymes based on cyt c.

Recently, we have been exploring a new route to novel metalloenzymes which involves the construction of hybridized protein scaffolds from different metalloproteins. We have previously reported that some functional metalloproteins could be generated via hybrids of cyt c and cytochrome P450 (CYP450) (Ying et al., 2011). In this regard, we further discovered that cyt c and peroxidase are ideal candidates for hybridization as they possess the same proximal axial heme ligand (histidine), and also the same catalytic intermediate, named Compound I, an active ferryl species (Fe(IV) = O) paired with a porphyrin free radical cation (Hiner et al., 2002; Pierattelli et al., 2004; Gumiero et al., 2011). The major difference between the heme environment of cyt c and peroxidase comes from their distal heme region. Several residues are highly conserved (such as His, Arg, Phe or Trp) in the distal heme pockets of most peroxidases (Dunford and Stillman, 1976; Smith and Veitch, 1998), as shown in Fig. 1a and b (right). These residues are utilized to facilitate the formation of Compound I in the catalytic cycle and, therefore, are considered to be key requirements for the catalytic activity of peroxidase. In comparison with a peroxidase, cyt c also possesses an axial histidine ligand but lacks the distal catalytic residues (Fig. 1b). The distal residues in cyt c (residues 71–85) form an omega loop, which facilitates the biological electron transfer via the axial ligand Met80. Mutating the Met80 and introducing new residues into the distal loop of cyt c will certainly abolish its function in cellular respiration; however, it is possible that some new functionality can be generated while the good stability of cyt c conserved. To introduce the peroxidase-like distal histidine, we previously developed the cyt c P71H mutant, but found it did not show any peroxidase activity (Lan et al., 2011). However, the unusual His–His axial coordination of heme iron in the cyt c P71H variant has provoked intense interest in understanding the structure–activity relationships modulated by the distal heme region, and interest in further engineering using other strategies to introduce peroxidase activity into cyt c.

In this study, we generated three ‘hybrid’ proteins of cyt c and peroxidase via structure-based rational mutagenesis of cyt c. Four residues in the distal heme region of cyt c were substituted, which involved positions 67 (Tyr67 to Arg), 70 (Asn70 to Phe/Trp/Ala), 71 (Pro71 to His) and 80 (Met80 to...
The heme pocket of peroxidase was hence introduced into cyt c, in order to eliminate the Met80–heme coordination). The heme pocket of peroxidase was hence introduced into cyt c through this simple approach. The resulting variants were named HY-N70A (the multiple mutant Y67R/N70A/P71H/M80V), HY-N70F (the Y67R/N70F/P71H/M80V mutant) and HY-N70W (the Y67R/N70W/P71H/M80V mutant), respectively. The UV-Vis and EPR spectra of the variants were investigated, and the stability and peroxidase activity were examined by acid denaturation and stopped-flow kinetics. The variants were found to exhibit high peroxidase activity and conserve the impressive stability of cyt c.

Materials and methods

Materials

The plasmid pBTR1 was a generous gift from Professor A.G. Mauk. Pfu DNA polymerase was purchased from New England Biolabs. QIAprep Spin Miniprep Kit was the product of Qiagen. QuikChange Site-Directed Mutagenesis Kit was purchased from Stratagene. The Escherichia coli strain BL21(DE3)pLysS was obtained from Novagen. CM-52 and CM Sepharose Fast Flow resin were purchased from Whatman and Pharmacia, respectively. All other reagents were of analytical grade.

Gene construction

The original pBTR1 plasmid, which encodes genes of the yeast iso-1-cytochrome c (CYC1) and yeast cytochrome c heme lyase (CYC3), has the native Cys102 residue substituted by a threonine residue to prevent formation of disulfide bonds between cyt c molecules, and also contains a mutation of K72A to prevent this residue to serve as a ligand in the alkaline form of the protein (Pollock et al., 1998; Rosell and Mauk, 2002). For the purpose of this work, this variant was used as the reference cytochrome and was referred to wild-type protein. The mutants were constructed based on the pBTR1 plasmid with the QuickChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s protocol. The plasmid was polymerase chain reaction amplified in the elongation process using PfuTurbo DNA polymerase according to the manufacturer’s protocol. The incorporation of the two oligonucleotides primers generates the mutated plasmid containing stagger nicks. The product was then treated with DpnI restriction enzyme, specific for methylated and hemimethylated DNA, then the parental DNA template was digested (almost all DNA isolated from commonly E.coli strains is usually dam methylated). The nicked vector DNA containing the desired mutations was then transformed into XL10-Gold competent cells. The plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen) and the identity of the mutants was confirmed by DNA sequencing.

Protein expression and purification

Expression and purification of the variants were performed according to the methods described previously (Ying et al., 2009b) with modifications. Cultures were initiated with a single, freshly transformed E.coli Rosetta(DE3)pLysS that served as the inoculum for 50 ml SB media supplemented with 100 mg ml⁻¹ ampicillin to 0.1% (v/v). After overnight incubation at 37°C with vigorous shaking (300 rpm), 5 ml was used to inoculate 800 ml modified SB (tryptone: 10 g l⁻¹, yeast extract: 8 g l⁻¹, sodium chloride: 5 g l⁻¹, glycerin: 1.5 ml l⁻¹, sodium nitrate: 4 g l⁻¹) with 100 mg l⁻¹ ampicillin in a 1 l flask and were incubated for 12–15 h. The cells were harvested by centrifugation (Sorvall SLA-3000 rotor, 4°C, 5000 rpm, 15 min) and resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 3 g l⁻¹ lysozyme and a few crystals of DNase I and RNase A. The mixture was stirred continuously for 1 h at 4°C, and then lysed by sonication on ice. This lysate was cleared by centrifugation (Sorvall SS-34 rotor, 4°C, 15 000 rpm, 20 min), and the resulting solution was collected. The pellet was resuspended in the lysis buffer and centrifugated repeatedly until the pellet and the supernatant were no longer pink. The colored fractions were pooled, and (NH₄)₂SO₄ (15% w/v) was added over a period of 30 min while stirring gently at 4°C. The resulting suspension was centrifuged before dialyzing the supernatant fluid overnight in distilled water. The dialysate was centrifuged, and the protein in the cleared solution was loaded on a column of CM-52 cation exchange resin equilibrated with Buffer A (50 mM sodium phosphate, pH 6.7). After loading, the column was washed with two volumes of Buffer A followed by two volumes of Buffer B (Buffer A plus 75 mM NaCl). Then the protein was eluted with Buffer C (Buffer A plus 250 mM NaCl) and exchanged into Buffer A by repeated ultrafiltration with an YM-10 membrane (Amicon). Samples were oxidized with K₃[Fe(CN)₆] immediately prior to the final purification by cation exchange chromatography with a CM Sepharose Fast
Flow column equilibrated with sodium phosphate buffer (20 mM, pH 7.0). The protein was eluted with a linear NaCl gradient of 1–300 mM ml\(^{-1}\). Fractions with the \(R\)-value (\(A_{408}/A_{280}\)) > 5 were pooled, concentrated and exchanged into Buffer A prior to snap-frozen in liquid nitrogen and stored at \(-80^\circ\)C.

**Mass spectroscopy**

Molecular weight of the proteins was measured by electrospray ionization mass spectrometry (ESI-MS) using a Bruker Esquire 3000 Electrospray Mass Spectrometer (Bruker Daltonics, Germany). The protein solutions were first desalted by ultrafiltration or dialysis, and then dissolved in 10% formic acid (v/v).

**UV-Visible spectroscopy**

The UV-Visible spectra of the variants were recorded on a Hewlett-Packard 8453 diode array spectrometer equipped with a Neslab RTE-111 water circulator, controlled by laboratory software. Proteins were dissolved in 100 mM sodium phosphate buffer, pH 7.0. Protein concentrations were determined using the pyridine hemochromate method. The reduced samples were prepared by the addition of sodium hydrosulphite.

**EPR spectroscopy**

Samples were analyzed by X-band EPR on a Bruker EMX EPR spectrometer fitted with a Hewlett Packard 5352B microwave frequency counter. A standard Bruker cavity (ER4102ST) was used in conjunction with an Oxford Instruments ER910A cryostat for low-temperature analysis. The temperature for the samples was 10 K; the buffer was 20 mM in sodium phosphate (pH 7.0); the microwave power was 2.02 mW.

**pH titration**

Protein samples were prepared in the mixture of sodium phosphate and sodium acetate buffers ([protein] \(\approx\) 10 \(\mu\)M, ionic strength = 0.1 M), and titrated with increasing amounts of 10 \(M\) HCl at 25°C. The progress of acidification was monitored by a Hewlett-Packard 8453 spectrometer. The pH values were measured directly in the cuvette using a Schott microelectrode (type 16PH) connected to an Orion pH meter (type 310P-02).

**Stopped-flow kinetics**

The steady-state kinetics of oxidation of guaiacol were studied with an SF-61 DX2 stopped-flow apparatus (Hi-Tech, UK) thermostated at 25.0 ± 0.1°C. The \(\text{H}_2\text{O}_2\) solution was prepared with 30% stock solution and its concentration was determined with an absorption coefficient of 39.4 \(M^{-1}\) cm\(^{-1}\). Fractions with the \(R\)-value (\(A_{408}/A_{280}\)) > 5 were pooled, concentrated and exchanged into Buffer A prior to snap-frozen in liquid nitrogen and stored at \(-80^\circ\)C.

**Results**

**Protein preparation**

We have recently developed a highly effective expression system for yeast iso-1 cyt c, human cyt c and their mutant proteins (Ying et al., 2009a,b). Therefore, although four conserved residues of cyt c were mutated in this work, all the resulting variants were expressed at a high level. Through the procedures described in the ‘Materials and methods’ section, we successfully obtained the highly purified variants with the yield of \(~12\) mg \(l^{-1}\) culture. The purity of the variants was verified by ESI-MS spectra, in which the mass of HY-N70A was calculated to be 12 564.9 ± 0.5 (the theoretical mass is 12 565.6), HY-N70F 12 639.8 ± 0.5 (theoretical 12 641.7) and HY-N70W 12 679.0 ± 1.0 (theoretical 12 680.7) (data not shown). The purified wild-type c was found to be in the reduced state (pink color), while all the variants prepared using the identical protocol were in the oxidized states (dark red color). This suggests that the redox midpoint potentials of the variants dropped to a very low level due to the mutation of Met80, a key residue for the \(in\) \(vivo\) electron-transfer function of cyt c.

**Unique spectral characteristics of the variants**

The electronic absorption spectra of the oxidized and reduced forms of the variants are shown in Fig. 2a–c. The three proteins exhibit almost identical absorption spectra, indicating that they possess quite similar coordination environment in the heme pocket. The absorption maxima were summarized in Table I. Specifically, the spectra displayed the Soret band at 407 nm, the \(\alpha\) + \(\beta\) bands at \(~530\) nm in the oxidized form, while the Soret band at 417 nm, \(\beta\) band at \(~520\) nm and \(\alpha\) band at \(~550\) nm in the reduced form. Although it is certain that profound changes would be introduced into the distal heme region due to the Met80 mutation, the optical features of the variants are similar to those of the wild-type cyt c (Fig. 2d), except for the disappearance of a weak charge-transfer band centered at 695 nm, which is characteristic of heme–Met80 coordination. Surprisingly, a small peak centered at \(~670\) nm was found in Fig. 2b or Fig. 2c. In the HY-N70A variant, this peak was slightly blue shifted to 655 nm (Fig. 2a). Such peaks, to the best of our knowledge, have never been reported before in the mutagenesis studies of cyt c. Interestingly, this behavior was reminiscent of the five-coordinated heme, which possesses a characteristic small band at 620 nm (Lo et al., 1995; Silkstone et al., 2005). Thus, although the 620 nm peak was not found in the variants, indicating the heme is primarily in the six-coordinated low spin state, we presume that the sixth ligand is probably a labile one, reflected by the 655 and 670 nm peaks.

The heme environment of the variants was further investigated via EPR studies. As shown in Fig. 2e, the EPR signals at \(g_x = 2.93\), \(g_y = 2.29\) and \(g_z = 1.53\) clearly demonstrate that the variants possess an unusual His–His heme coordination. There is only one histidine residue, His71, in the distal heme pocket. Thus, the two ligands probably come from the proximal His18 and distal His71. This observation is in agreement with the NMR data of the cyt c P71H mutant, in which the His71 was found to be bound to heme (Ying et al., 2010). Combined with the UV-Vis spectra (Fig. 2a–c),
it is evident that His71 is loosely bound to heme in the distal pocket, replacing the Met80 ligand in the wild-type cyt c. The UV-Vis spectral characteristic of HY-N70A (655 nm) is more close to five-coordinated (620 nm) compared with other variants which possess larger side chain groups, showing that the His71–heme ligation is possibly tuned by the introduced Arg67 and the amino acid at residue 70, and this ligation in HY-N70A is probably a bit looser than that in HY-N70F and HY-N70W.

**Stability of the variants**

The electronic spectrum of ferric cyt c exhibits a significant pH-dependence behavior. With increasing pH cyt c would undergo a conformational change called alkaline conformational transition (Looze et al., 1978; Hoang et al., 2003; Martinez and Bowler, 2004; Ying et al., 2009b). During this transition protein structure remains intact while the Met80 ligand would be replaced by lysine. Multiple conformational changes could also be observed during the urea denaturation...
Cyt c. Besides, cyt c possesses superior thermal stability that can keep intact even heated to 80°C (Sorrell and Martin, 1989). Thus, the stability of cyt c is evaluated by acid denaturation. Herein, the stabilities of three variants toward acid denaturation were tested, as shown in Fig. 3.

For the three variants, the absorption spectra did not show any change with the addition of 10 M HCl from pH 7 to pH 4. With further decreasing pH, the Soret band was gradually blue shifted from 407 to 395 nm, reflecting that the protein was partially unfolded. Coincidently, the 620 nm peak appeared, showing that the His71–heme ligation was removed and the high-spin heme was generated. The Soret band absorption was plotted with decreasing pH, as shown in Fig. 3b. The apparent pK value (pKₐ) calculated from this curve could reflect the protein stability toward acid denaturation. The pKₐ for HY-N70A is 3.1, HY-N70F is 3.0 and HY-N70W is 2.9. Interestingly, HY-N70A, which possesses a bit looser His71–heme coordination as discussed above, is slightly less stable than the other two variants. The pKₐ values of the three proteins are quite similar to that of the wild-type cyt c (3.0, Fig. 3b), indicating that the variants retain the superior stability of the wild-type protein.

### Peroxidase activity measurement

Guaiacol, a typical substrate of peroxidase, was selected for the enzymatic analysis. The initial rate of guaiacol oxidation was measured by following the formation of colored oxide, tetra-guaiacol. The steady-state kinetic constants kₑ and Kₘ were calculated from initial velocity studies by the Michaelis–Menten equation. As shown in Fig. 4, the kinetic data of the three variants and the wild-type cyt c can be well fitted using the Michaelis–Menten equation, indicating that the peroxidation reactions catalyzed by the variants also follow the classical catalysis mechanism. The kinetics parameters were summarized in Table II. The kₑ values of the wild-type cyt c, HY-N70A, HY-N70F and HY-N70W were calculated to be 0.014, 0.016, 0.012 and 0.017 mM, while kₑ were calculated to be 0.6, 3.6, 2.4 and 1.3 s⁻¹, respectively. Consequently, the kₑ/Kₘ value of HY-N70W (77 000 M⁻¹ s⁻¹), representing the catalytic efficiency, is almost twice higher than that of the wild-type cyt c (43 000 M⁻¹ s⁻¹), while the kₑ/Kₘ value of HY-N70A (225 000 M⁻¹ s⁻¹) or HY-N70F (200 000 M⁻¹ s⁻¹) is about

### Table I. Absorption, EPR and acid denaturation properties of cyt c and the variants

<table>
<thead>
<tr>
<th></th>
<th>Oxidized</th>
<th>Reduced</th>
<th>EPR (gₓ, gᵧ, gₓ)</th>
<th>Acid denaturation pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt c</td>
<td>410, 530, 695</td>
<td>416, 521, 550</td>
<td>—</td>
<td>3.0</td>
</tr>
<tr>
<td>HY-N70A</td>
<td>407, 529, 655</td>
<td>417, 522, 550</td>
<td>2.93, 2.29, 1.53</td>
<td>3.1</td>
</tr>
<tr>
<td>HY-N70F</td>
<td>407, 531, 669</td>
<td>417, 521, 550</td>
<td>2.93, 2.29, 1.54</td>
<td>3.0</td>
</tr>
<tr>
<td>HY-N70W</td>
<td>407, 531, 669</td>
<td>417, 521, 550</td>
<td>2.93, 2.29, 1.53</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Fig. 3. Stability of the variants. (a) The acid titration of the ferric form of HY-N70A. (b) Absorbance changes at 407 nm as a function of pH values for the variants and wild-type cyt c. A colour version of this figure is available as supplementary data at PEDS Online.

Fig. 4. Steady-state rate of the peroxidation as a function of guaiacol concentration for the wild-type cyt c and the variants.
five-fold higher. It is obvious that the variants possess elevated peroxidase activity compared with the wild-type protein. It is also interesting to note that the $k_{\text{cat}}/K_m$ values of the variants are even higher than those of the native peroxidases. The $k_{\text{cat}}$ of lignin peroxidase, a typical peroxidase, was reported to be $7.7 \text{ s}^{-1}$, which is more than twice as high as the cyt $c$ variants (Koduri and Tien, 1995). However, the affinity of lignin peroxidase to substrates ($K_m = 0.16 \text{ mM}$) was 10 times lower than that of the cyt $c$ and variants (Koduri and Tien, 1995). Compared with the distal heme region of the peroxidases, that of the cyt $c$ is formed by a flexible loop instead of rigid helices (Fig. 1b). It is possible that such loops are favorable for the entry and/or binding of substrates.

**Discussion**

One of the fundamental purposes in protein engineering is to develop stable enzymes for industrial applications. However, it has long been realized that protein stability and enzymatic activity are usually reciprocally related (Shoichet et al., 1995; Beadle and Shoichet, 2002). Rational mutagenesis studies that endeavor to enhance catalytic property of an enzyme often lead to a significant reduction in its stability. Therefore, the endeavor to enhance catalytic property of an enzyme often lead to a significant reduction in its stability. Consequently, the distal Arg67 in the variants could probably be used to help formation of Compound I. In addition, or alternatively, it is possible that the labile His71 ligand could also serve as a distal histidine and fulfill such mission. Support for this view may be provided by the fact that HY-N70A, which possibly possesses the least stable His71–heme ligation among the variants, exhibits the highest peroxidase activity. Evidently, using a simple strategy that introduces highly conserved residues into the distal heme pockets of peroxidases into the scaffold cyt $c$, we successfully obtained three ‘hybrids’ (multiple mutants), which exhibit high peroxidase activity and retain the stability of cyt $c$.

Some interesting biological consequences emerged from these studies. Firstly, the coordination of His71 to heme, as well as a remarkable perturbation in the heme pocket of the variants, suggests that the distal heme region (residues 71–85 omega loop) of cyt $c$ is quite flexible. Interestingly, this omega loop was recently found to be one of the fastest unfolding units in cyt $c$ (Bai et al., 1995; Krishna et al., 2003), and coincidently underwent major conformational changes when cyt $c$ was involved in different biological functions beyond respiration (Baddam and Bowler, 2005a,b; Kagan et al., 2005; Sinibaldi et al., 2008). We have also previously reported that this omega loop was driven away from the heme during a period of unusual rapid protein evolution in the primate lineage (Ying et al., 2009b). Taken together, it is distinct that the residues 71–85 loop is critical for the structure and function of cyt $c$. We assume that this flexible region in cyt $c$ facilitates its different conformational transitions in vivo, and thus facilitates its biological multi-functions. Besides, the His71 coordination is reminiscent of the alkaline conformation of cyt $c$, in which the axial ligand Met80 is replaced by Lys72, Lys73 or Lys79 at alkaline pH (Rosell and Mauk, 1998). Since there is no crystal structure of the alkaline form of cyt $c$, it is reasonable that the structural characterization of the His71-variants, which could possibly serve as the model for alkaline conformer, would contribute to the further understanding of the conformation–function relationship of cyt $c$.

In conclusion, we have generated three novel hybrid metalloenzymes by introducing highly conserved residues in the catalytic pockets of peroxidases into cyt $c$. The multiple mutants were found to exhibit high peroxidase activity and retain the impressive stability of cyt $c$. In fact, this work provides a rare example where hybrids could inherit the advantages of both parent proteins. We expect that our strategy could be extended to other cases of metalloprotein engineering, and lead to the development of stable and active biocatalysts which could open the door to a wide range of industrial and biomedical applications.

**Supplementary data**

Supplementary data are available at PEDS online.

**Acknowledgments**

We thank Dr A.G. Mauk for the yeast iso-1 cytochrome $c$ plasmid.

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**Table II. Kinetics parameters of guaiacol oxidation**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt $c$</td>
<td>0.6</td>
<td>14</td>
<td>43 000</td>
</tr>
<tr>
<td>HY-N70A</td>
<td>3.6</td>
<td>16</td>
<td>225 000</td>
</tr>
<tr>
<td>HY-N70F</td>
<td>2.4</td>
<td>12</td>
<td>200 000</td>
</tr>
<tr>
<td>HY-N70W</td>
<td>1.3</td>
<td>17</td>
<td>77 000</td>
</tr>
<tr>
<td>Lignin peroxidase (Koduri and Tien, 1995)</td>
<td>7.7</td>
<td>160</td>
<td>48 000</td>
</tr>
</tbody>
</table>
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**References**


