Quaternary structure of quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* and its reoxidation with a novel cytochrome c from this organism

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Quinoprotein (2,7,9-tricarboxy-1H-pyrrolo-[2,3-f]quinoline-4,5-dione quinone form (PQQ)-containing) ethanol dehydrogenase (EDH) from *Pseudomonas aeruginosa* ATCC 17933 was purified to homogeneity. EDH has an α₂β₂ configuration and subunits comparable in size to those of methanol dehydrogenase (MDH). Compared with other PQQ-containing dehydrogenases, Ca²⁺ is rather loosely bound and it seems necessary for PQQ binding and stability of EDH. Two soluble cytochromes c were detected in extracts from ethanol-grown cells and both were purified. One of these has an α-band at 551 nm for its reduced form, the oxidized form being an excellent electron acceptor for the semiquinone form of EDH. Since this cytochrome is quite different from the already known cytochrome c₅₅₁ (operating in nitrate respiration) of this organism, it is indicated here as cytochrome c₉₆₈. Comparison of the N-terminal amino acid sequence of cytochrome c₉₆₈ with the complete sequence of cytochrome c₉ (the electron acceptor of MDH), cytochrome cₕ (the electron acceptor of cytochrome cₙ) and cytochrome c₅₅₁ revealed some similarity only to internal stretches of amino acids of the last two. The other soluble cytochrome appeared to be the already-known cytochrome c₅₅₁. Since it was not an electron acceptor for cytochrome c₉₆₈ (neither for EDH), cytochrome cₙ is lacking in the quinoprotein-EDH-ethanol oxidation system of *P. aeruginosa*. It seems, therefore, that the respiratory chains for MDH and EDH are different.

**INTRODUCTION**

Gram-negative, methane- or methanol-utilizing bacteria oxidize methanol via quinoprotein (PQQ-containing) methanol dehydrogenase (EC 1.1.99.8, MDH). The enzyme has an α₂β₂ structure with subunits of 60 and 10 kDa [1,2]. Electron transfer from the fully- and half-reduced forms of MDH occurs to a specific, soluble, haem c-containing cytochrome, commonly indicated as cytochrome cₙ. Reduced cytochrome cₙ becomes subsequently oxidized by a cytochrome named cytochrome c₉₆₈ [3].

Ethanol-grown *Pseudomonas aeruginosa* contains an NAD-dependent as well as a quinoprotein alcohol dehydrogenase [4]. The latter enzyme, which has been named ethanol dehydrogenase (EDH), has been purified from *P. aeruginosa* [5,6] and *P. putida* [7]; B. W. Groen and J. A. Duine, unpublished work) and its quinoprotein nature established [5]. EDH and MDH appear to have properties in common: the requirement for a high pH and the presence of ammonia or primary amines as activators in the *in vitro* assay with artificial electron acceptors [8]; the shape and maxima of their absorption spectra and the effect of the suicide inhibitor cyclopropenone, suggesting a similar catalytic role for PQQ in both enzymes [5,9] and the presence of Ca²⁺ [10,11]. To enable further comparison this paper describes studies which attempt to elucidate the quaternary structure and to detect the electron acceptor for EDH.

**MATERIALS AND METHODS**

**Organism and growth conditions**

*P. aeruginosa* LMD 89.1 (ATCC 17933) was cultured aerobically in a 20 l fermentor at 37 °C on a mineral medium [12] with 0.5 % ethanol as a carbon and energy source. The pH was maintained at 7.0 with 3 M KOH, the airflow was 3 l/min and the vessel was stirred at 300 rev./min. Cells were harvested at the end of the exponential growth phase (A₉₀₀ - A₆₀₀ 1.0). The yield was 1.7 g cells (wet weight)/l. Cell paste was stored frozen.

**Isolation of ethanol dehydrogenase and soluble cytochromes c**

Cell paste (30 g) was suspended in an equal volume of 10 mM Mops buffer, pH 7.0 (buffer A). After adding DNAase to the suspension, the cells were disrupted twice in a French pressure cell at 110 MPa and the suspension was centrifuged for 10 min at 16300 g. Protein in the supernatant precipitating at 40 °C and 40–80 % (NH₄)₂SO₄ saturation was collected by centrifugation at 48200 g for 20 min. The 40–80 % precipitate was dissolved in a minimal amount of buffer A and dialysed against the same buffer.

The dialysed preparation was loaded on a DEAE-Sephrose column (Pharmacia) (10 cm × 5 cm) equilibrated with 10 mM Mops buffer, pH 7.0, containing 10 mM CaCl₂ (buffer B). Ethanol dehydrogenase and cytochrome c₅₅₁ are not retained under these conditions and were collected in a total volume of 100 ml. The bound cytochrome c₉₆₈ was eluted with a linear NaCl (1 M) gradient in buffer B in 60 min at a flow rate of 2 ml/min at a concentration of 0.4 M NaCl in a fraction of 70 ml. This was brought to 3 M in ammonium acetate and subjected to hydrophobic-interaction chromatography on a Fractogel TSK Butyl-650 (S) column (Merck) (15 cm × 3 cm) equilibrated with buffer B and containing 3 M ammonium acetate. The column was eluted with a linear gradient from 3.0 M to zero ammonium acetate in buffer B in 60 min at a flow rate of

Abbreviations used: PQQ, the quinone form of 2,7,9-tricarboxy-1H-pyrrolo-[2,3-f]quinoline-4,5-dione; MDH, methanol dehydrogenase; EDH, ethanol dehydrogenase; EDH₉₆₈, EDH in the semiquinone state; EDH₀ₓ₆₈, fully oxidized form of EDH.

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1 ml/min. Cytochrome $c_{\text{EDH}}$ was eluted at a concentration of 0.8 M ammonium acetate. After concentrating the fraction with a Centricon-10 concentrator (Amicon) to 2 ml, cytochrome $c_{\text{EDH}}$ was applied to a Superdex 75 gel-filtration column (Pharmacia) equilibrated with 0.1 M sodium phosphate buffer, pH 6.5. Chromatography was carried out by eluting with the same buffer at a flow rate of 0.5 ml/min. The cytochrome $c_{\text{EDH}}$-containing fractions were pooled and dialysed against buffer A.

After adding ammonium acetate (3 M final concentration) to the solution containing EDH and cytochrome $c_{\text{EDH}}$, hydrophobic-interaction chromatography was carried out as described for cytochrome $c_{\text{EDH}}$. EDH and cytochrome $c_{\text{EDH}}$ were eluted at concentrations of 1.0 and 0.2 M ammonium acetate, respectively. After concentrating, each protein was subjected to gel-filtration chromatography by injecting on a Superose 12 gel-filtration column (Pharmacia), equilibrated with 10 mM Mops buffer, pH 7.0, containing 10 mM CaCl$_2$ and 0.1 M NaCl. The EDH-containing fractions were dialysed against buffer B and the cytochrome $c_{\text{EDH}}$ fractions against buffer A.

In all cases, the purification was carried out at room temperature and the final preparations were stored at $-80 \, ^\circ\text{C}$.

**Enzyme assays**

EDH activity was measured by following the reduction rate of Wurster’s Blue ($\text{NNN'N’-tetramethyl-p-phenylenediamine free radical}$ [13]), at 612 nm ($\epsilon_{\text{dd}} = 12.7 \, \text{mM}^{-1}\cdot \text{cm}^{-1}$). For that purpose, 33 $\mu\text{l}$ 0.3 mM Wurster’s Blue, 900 $\mu\text{l}$ of 0.1 M sodium borate buffer, pH 9.0, containing 50 mM ethylenediamine and 1 mM ethanol and 5 $\mu\text{l}$ of 0.2 M KCN were mixed. The reaction was started by adding 100 $\mu\text{l}$ of enzyme solution. One enzyme unit is defined as the amount reducing 1 $\mu\text{mol}$ Wurster’s Blue per min at 25 $^\circ\text{C}$.

**Protein determinations**

Protein concentrations during the purification were determined by the method of Bradford [14] with bovine serum albumin as a standard. Absorption coefficients of the purified proteins were determined according to van Iersel et al. [15] using the equation:

$$A_{280}^{\text{obs}} = 34.14A_{280}/A_{205} - 0.02$$

**Isoelectric point and molecular mass determinations**

The molecular mass of native EDH was determined by gel filtration on a Superose 12 column equilibrated with 0.1 M sodium phosphate buffer, pH 6.5, using ferritin, $\gamma$-globulin (150 kDa), yeast alcohol dehydrogenase (140 kDa), bovine serum albumin (67 kDa), cytochrome $c$ from horse heart (13 kDa) and potassium ferricyanide as markers. The subunit composition and content of EDH were determined with the same column, except that 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1% (w/v) sodium dodecylsulphate (SDS) and 0.1 M NaCl was used for equilibration and elution. For that purpose, 100 $\mu\text{l}$ of EDH solution (7.2 mg/ml) was mixed with 400 $\mu\text{l}$ of 0.1 M sodium phosphate buffer, pH 6.5, containing 1.0% (w/v) SDS and 0.1 M NaCl after which the mixture was heated for 2 min at 100 $^\circ\text{C}$. After cooling and centrifugation, 100 $\mu\text{l}$ of the supernatant was injected on the column. The eluate was monitored by a photodiode array detector. The molar ratio of the $\alpha$ and $\beta$ subunits of EDH was calculated by dividing the surface area of their peaks in the chromatogram (taken at 205 nm) by the respective molecular masses of the subunits.

The molecular mass of the native cytochromes $c$ was determined by electrophoresis on gradient polyacrylamide (8–25%) gels with the Phast system equipment (Pharmacia). The gels were calibrated with the high molecular mass marker kit (Pharmacia). The molecular mass of the subunits was determined in the same way except that 1% (w/v) SDS was included in the buffer. The proteins were denatured for 2 min at 100 $^\circ\text{C}$ and the low molecular mass marker kit was used for calibration.

The isoelectric points of the proteins were determined by isoelectric focusing in the pH range 3–9. The isoelectric focusing marker kit (Pharmacia) was used for calibration.

Protein staining was performed with Coomassie Brilliant Blue R250 and detection of cytochromes $c$ also by haem staining with 3,3',5,5'-tetramethylbenzidine (Janssen Chimica) [16].

**Midpoint potential of the cytochromes**

Potentiometric titrations were performed as described by van Wielink et al. [17]. Cytochrome $c_{\text{EDH}}$ (5.0 $\mu\text{M}$) was titrated in 0.1 M Hepes buffer, pH 7.0, in the presence of 0.1 M quinhydrone ($E'_q = +280 \, \text{mV}$ (Merck)), 0.4 M diaminozuridine ($E'_q + 275 \, \text{mV}$) (Fluka Chemie), 0.2 mM 2,5-dimethyl-p-benzoquinone ($E'_q = +180 \, \text{mV}$) (ICN Pharmaceuticals) and 0.1 M trimethylhydroquinone ($E'_q = +115 \, \text{mV}$) (ICN Pharmaceuticals).

**N-terminal amino acid sequence determination**

The amino acid sequence of the N-terminal part of cytochrome $c_{\text{EDH}}$ was determined by Edman degradation with a gas-phase amino acid sequenator [18] at the Laboratory for Medical Biochemistry, Sylvius Laboratories, Leiden, The Netherlands. Comparison of the sequence with that of other proteins was carried out by using the data in the Swissprot-, the GenPept-, the Gen-bank and the EMBL-data libraries [19].

**Stopped-flow kinetics**

Kinetic experiments were performed at 20.0 $^\circ\text{C}$ with a HI-Tech SF 50 stopped-flow spectrophotometer. Data acquisition was performed with a 100 kHz DASH 16F A/D converter controlled by an Olivetti M24 SP computer. Reduction of ferricytochrome $c_{\text{EDH}}$ by the semiquinone form of EDH (EDH$_{\text{sem}}$) was monitored at 418 nm (giving the highest difference in absorbances between ferri- and ferro-cytochrome $c_{\text{EDH}}$).

Pseudo-first-order rate constants were calculated by non-linear regression with the use of a Gauss–Newton algorithm, available with ASYST (Keithley). The data used were the average of at least three measurements. Experiments were carried out by diluting the EDH$_{\text{sem}}$ preparation with 10 mM Mops buffer, pH 7.0, and mixing this with an equal volume of cytochrome $c_{\text{EDH}}$, diluted in the same buffer. Concentrations of EDH$_{\text{sem}}$ mentioned in the text are the final concentrations of subunit after mixing.

**RESULTS AND DISCUSSION**

**Purification and properties of ethanol dehydrogenase**

A purification scheme was elaborated for EDH (Table 1) and its potential electron acceptors, the soluble cytochromes $c$. The final EDH preparation appeared to be homogeneous, as judged from electrophoresis and gel filtration (the identity of the peak was checked by comparing the absorption spectra). Comparison of the shape of the absorption spectrum with those of the different
Table 1  Purification of quinoprotein ethanol dehydrogenase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract*</td>
<td>3651</td>
<td>2357</td>
<td>0.65</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>2411</td>
<td>1849</td>
<td>0.77</td>
<td>78</td>
<td>1.2</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>1109</td>
<td>1224</td>
<td>1.1</td>
<td>52</td>
<td>1.7</td>
</tr>
<tr>
<td>TSK Butyl-650 (S)</td>
<td>313</td>
<td>945</td>
<td>3.0</td>
<td>40</td>
<td>4.6</td>
</tr>
<tr>
<td>Superoxide 12</td>
<td>24</td>
<td>943</td>
<td>40</td>
<td>40</td>
<td>62</td>
</tr>
</tbody>
</table>

* Originating from 30 g cell paste.

Figure 1  Gel filtration of denatured EDH

A sample of EDH (7.2 mg/ml) in 0.1 M sodium phosphate buffer, pH 6.5, containing 1% SDS and 0.1 M NaCl, was heated at 100 °C for 2 min and applied to a Superose 12 column in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1% SDS and 0.1 M NaCl. Elution occurred at a flow rate of 0.2 ml/min and the eluate was monitored at 205 nm.

The soluble cytochromes c

Soluble cytochromes c were only detected in the 40–80% (NH₄)₂SO₄ fraction, not in the 40% precipitate (only minor amounts of membrane-bound cytochromes were present) nor in the 80% supernatant (devoid of any spectral peaks reminiscent of cytochromes). Upon purification of the 40–80% precipitate, it appeared to contain two soluble cytochromes c, as demonstrated by the typical spectra of the final preparations and the positive response to tetramethylbenzidine staining after electrophoresis of the denatured samples. Moreover, probably a copper protein is present also since blue-colored fractions were obtained from the hydrophobic-interaction chromatography column after cytochrome c₉₅₉ had eluted.

The preparation showing an absorption maximum at 551 nm on reduction is named here cytochrome cₑRH in view of its functioning as an electron acceptor to EDH (see below) and the differences from the previously-described cytochrome c₉₅₉ from P. aeruginosa (strain PA01161) (Table 2). The final preparation appeared to be homogeneous and to consist of a monomeric protein as the same molecular masses were found for native and denatured samples (14.5 kDa). Thus, cytochrome cₑRH is significantly larger than cytochrome c₉₅₉ (8.7 kDa). Since cytochrome cₑRH has the same functional role as cytochrome c₉₅₉, it was interesting to see whether overall similarity existed. As shown in Table 2, the properties of cytochrome cₑRH fall in the ranges observed for a number of cytochromes c₉₅₉. However, comparison

preparations with a high specific activity (40 unit/mg) which showed no increase upon CaCl₂ and PQQ addition, CaCl₂ was routinely added to the buffers. The results show that in contrast to other PQQ-containing enzymes, Ca²⁺ in EDH is loosely bound, its detachment leading to loss of PQQ and lability of the apo-enzyme.

Gel filtration indicated a molecular mass of 136 ± 12 (n = 3) kDa for native EDH. Polycrylamide gel electrophoresis of denatured EDH (approximately 4 μg per lane) in the presence of SDS revealed two bands, one with a molecular mass of 60 kDa and the other of 9 kDa. This finding contradicts previous reports [5,6] where only one band was observed. However, as the two bands were consistently found in electrophoresis of active preparations and peaks corresponding to the same size were also observed in the gel-filtration chromatogram of denatured EDH (Figure 1), we conclude that EDH contains α and β subunits with similar sizes to those reported for MDH [1,2]. To explain the contradictory findings, the β subunit is much smaller in size than the α subunit and, as a consequence, the intensity of the bands from denatured EDH observed after electrophoresis and protein staining will differ (7-fold if both have the same dye-affinity) such that the β subunit will be easily lost or not visualized under inappropriate conditions. This may be why the β subunit of MDH has been overlooked for more than 20 years by many research groups [1] and it may also apply to EDH for the two cases reported [5,6].

On integrating the surface areas of the peaks in the chromatogram of denatured EDH (Figure 1), a value of 2.2 was found for the first (corresponding to the α subunit) and of 0.3 for the second one (corresponding to the β subunit). Since the chromatogram was taken at 205 nm and absorbance at this wavelength is directly related to the protein concentration [15], dividing the surface areas by the corresponding subunit molecular masses (60 and 9 kDa) gives figures related to the subunit concentration. From these experiments, a ratio of 1.0 ± 0.2 (n = 6) was found, indicating that EDH has an α₂β₂ composition, just as has been recently found for a number of MDHs [1,2,21].

redox forms of MDH [20] indicated that the final EDH preparation consisted of enzyme in its semiquinone form (EDHₙₙₑ). This was confirmed by similar A₂₆₅/A₂₈₀ ratios (6.3) and the fact that EDHₙₑ could be oxidized by ferricytochrome cₑDH.

Initially, purification was carried out using buffers without CaCl₂. The final preparation in that case had a specific activity of 2.4 unit/mg protein and was very labile upon storage at 4 °C. Upon addition of CaCl₂ (10 mM) to such a preparation, an increase in specific activity to 26 occurred and an additional increase occurred upon adding PQQ (10 μM). Since incorporation of CaCl₂ in the buffers prevented the lability and gave
of the N-terminal amino acid sequence (19 amino acids) of cytochrome c\textsubscript{EDH} with that of cytochrome c\textsubscript{L} from Methylbacterium extorquens AM1 showed no similarity at all. Although an internal stretch was found having some similarity (21%), internal stretches exist in cytochrome c\textsubscript{353} of \textit{P. aeruginosa} PA01161 [22,23] and cytochrome c\textsubscript{L} of \textit{Methylbacterium extorques} AM1 [3] having even higher similarity (42% and 32%, respectively) (Figure 2). Since the latter two contain the typical haem c-binding sites but the sequence of cytochrome c\textsubscript{EDH} does not, the similarity is questionable. In conclusion, cytochrome c\textsubscript{EDH} is structurally different from cytochrome c\textsubscript{AM} and the amino acid sequence of the N-terminal part also suggests structural dissimilarity to cytochrome c\textsubscript{L}.

The other coloured fraction, which showed an absorption maximum at 556 nm upon reduction, was also purified to homogeneity, as judged from the results of electrophoresis and gel filtration of the final preparation. Determination of the molecular mass of the native protein gave a value of 80 kDa, whilst that for the denatured protein was 40 kDa, indicating that it consists of two subunits of equal size. These characteristics are very similar to those of a cytochrome c\textsubscript{AM} isolated from anaerobically grown \textit{P. aeruginosa} PA01161, using nitrate as electron acceptor [24]. Since no other soluble cytochrome c was detected and soluble cytochrome c\textsubscript{L} is the electron acceptor for cytochrome c\textsubscript{L} in methylotrophic bacteria [25,26], we investigated whether cytochrome c\textsubscript{AM} could function as a cytochrome c\textsubscript{H}. However, it appeared that ferrocytochrome c\textsubscript{AM} was unable to oxidize ferrocytochrome c\textsubscript{EDH}. Since it was also unable to act as a terminal acceptor for EDH\textsubscript{sem}, a role of cytochrome c\textsubscript{AM} in ethanol oxidation via quinoprotein EDH is highly unlikely. The latter conclusion is in accordance with the observation that growth with substrate limitation (batch-fed on ethanol) gave cells which did contain EDH and cytochrome c\textsubscript{EDH} but no cytochrome c\textsubscript{AM}. The results indicate that a cytochrome c\textsubscript{AM}-like reoxidizable protein does not occur in ethanol-grown \textit{P. aeruginosa}. The blue-coloured protein fraction was able to oxidize ferrocytochrome c\textsubscript{EDH} (results not shown). In view of this and the tentative conclusions on the structural differences between cytochrome c\textsubscript{EDH} and cytochrome c\textsubscript{L}, of \textit{M. extorques} AM1, EDH seems to form part of a different type of respiratory chain from that in which MDH is operating.

**Table 2** Comparison of cytochromes c

<table>
<thead>
<tr>
<th>Properties</th>
<th>Cytochrome c\textsubscript{EDH}</th>
<th>Cytochrome c\textsubscript{AM}</th>
<th>Cytochrome c\textsubscript{L}</th>
<th>Cytochrome c\textsubscript{H}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass (kDa)</td>
<td>14.5</td>
<td>8.7</td>
<td>14–22</td>
<td>8.5–15</td>
</tr>
<tr>
<td>Absorption maxima (nm)</td>
<td>410</td>
<td>409</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced form</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorption coefficient of the reduced forms (mM cm\textsuperscript{-1})</td>
<td>19</td>
<td>29</td>
<td>551</td>
<td>161</td>
</tr>
<tr>
<td>At 551 nm</td>
<td>104</td>
<td>165</td>
<td>65–139</td>
<td>128–162</td>
</tr>
<tr>
<td>Absorbance ratios of the reduced forms</td>
<td>1.5</td>
<td>1.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A\textsubscript{551}/A\textsubscript{322}</td>
<td>0.5</td>
<td>1.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.8</td>
<td>4.6</td>
<td>3.5–4.9</td>
<td>4.5–9</td>
</tr>
<tr>
<td>Midpoint potential (mV)</td>
<td>+258</td>
<td>+286</td>
<td>+190–324</td>
<td>+224–373</td>
</tr>
</tbody>
</table>

* Data for cytochrome c\textsubscript{AM} of \textit{P. aeruginosa} PA01161 are from [22,23].
† Data for cytochrome c\textsubscript{L} and cytochrome c\textsubscript{H} of methylotrophic bacteria are from [3].

**Figure 2** Comparison of amino acid sequences of cytochromes c

Data (positions 139–157) for cytochrome c\textsubscript{AM} of \textit{P. aeruginosa} PA01161 are from [22,23], for cytochrome c\textsubscript{L} (positions 20–38) and cytochrome c\textsubscript{H} (positions 13–31) of \textit{M. extorques} AM1 from [3]. The typical haem c-binding amino acid residues [31] (CXXCH) are marked * for cytochrome c\textsubscript{AM} and cytochrome c\textsubscript{L}.

**Electron transfer between EDH\textsubscript{sem} and cytochrome c\textsubscript{EDH}**

Since EDH\textsubscript{sem} appeared to react very rapidly with ferrocytochrome c\textsubscript{EDH} in spectrophotometric experiments, stopped-flow experiments were carried out to measure the rate constant of electron transfer and the dissociation constant of complex formation. For that purpose the k\textsubscript{obs} (observed rate constant) was measured at 418 nm for varying EDH\textsubscript{sem} subunit concentrations at a constant ferrocytochrome c\textsubscript{EDH} concentration (0.75 \textmu M). Figure 3 shows that saturation kinetics exists for this reaction. The simplest reaction mechanism explaining this behaviour consists of two reaction steps:

\[
\text{EDH}_{\text{sem}} + \text{ferrocytochrome c}_{\text{EDH}} \\
\xrightarrow{k_{1,s}} \text{c}_{\text{EDH}}^\text{sem} + \text{ferricytochrome c}_{\text{EDH}} \\
\xrightarrow{k_{2,s}} \text{complex}
\]

In this scheme, it is assumed that k\textsubscript{2,s} is negligible, which seems justified since EDH will probably have a redox potential of +100 mV (such a value has been found for quinoprotein glucose dehydrogenase [27] and quinoprotein methylamine dehydrogenase [28] and the value of cytochrome c\textsubscript{EDH} is significantly larger [+258 mV (Table 2)]. Since the ferrocytochrome c\textsubscript{EDH}
concentration was kept constant, pseudo-first-order-rate conditions are present so that the steady-state approximation [29] can be applied to yield the following equation:

\[ k_{\text{obs}} = k_{14} [\text{EDH}_{\text{sem}}] / ([\text{EDH}_{\text{sem}}] + K_e) \]

in which

\[ K_e = (k_{-1} + k_{12}) / k_{11} \]

Using the experimental data, \( k_{14} \) and \( K_e \) were calculated by non-linear regression analysis. The value calculated for \( k_{14} \), (99.6 s\(^{-1}\)) is higher than that for MDH\(\text{sem} \) and ferricytochrome \( c_L \) (0.3–27 s\(^{-1}\)) [30]. Binding of ferricytochrome \( c_{\text{EDH}} \) to EDH\(\text{sem} \) is rather tight since the dissociation constant \( (K_e) \) is 5.7 \( \times 10^4 \) M, comparable to that for MDH\(\text{sem} \) and ferricytochrome \( c_L \) from Methylophaga marina \( (K_e = 5.0 \times 10^4 \text{ M}) \), unpublished results). In conclusion, the rates and affinity observed and the fact that no other redox protein was found having this activity make it very likely that cytochrome \( c_{\text{EDH}} \) is the natural electron acceptor of EDH in \( P. \) aeruginosa.

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