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INTRAPEPTIDE SEQUENCE HOMOLOGY IN RUBRERYTHRIN FROM *DESULFOVIBRIO VULGARIS*: IDENTIFICATION OF POTENTIAL LIGANDS TO THE DIIRON SITE

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Two regions in the amino acid sequence of the 21.5 kDa subunit of rubrerythrin from *Desulfovibrio vulgaris* (Hildenborough) are shown to be homologous. Rubrerythrin contains a non-heme, non-sulfur diiron site, and the internally homologous regions share homology with at least one proposed iron binding region of the component A α subunit of methane monooxygenase, which also contains a non-heme, non-sulfur diiron site. Comparison of the rubrerythrin sequences with those of the B2 subunit of *E. coli* ribonucleotide reductase, whose diiron site ligands have been identified, suggests that two glutamate and two histidine residues at positions 53, 56, 129, and 131 within the rubrerythrin sequence furnish ligands to the diiron site. A pair of EXXH sequences appears to represent a diiron binding motif in all three aforementioned proteins. No propene monooxygenase activity was detected with rubrerythrin using the assay designed to test activity of methane monooxygenase component A in the absence of other protein components.

Rubrerythrin (Rr) is a homodimeric non-heme iron-containing protein (native M_r = 43 kDa) of unknown function which was isolated from the sulfate reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) [1]. Each 21.5 kD subunit contains a rubredoxin-like iron-sulfur site in which four cysteinyl sulfur atoms are tetrahedrally coordinated to a single iron atom. One additional non-heme, non-sulfur diiron site was found for every *two* rubredoxin-like irons [1]. The Rr gene has recently been cloned and sequenced [2]. The C-terminal portion of the amino acid sequence derived from the nucleotide sequence shares some homology with the sequences of rubredoxins and, on this basis, the four cysteine ligands to the rubredoxin-like site can be readily identified. Eight His plus numerous Glu and Asp residues are also present, but their relative positions within the sequence show no apparent resemblance to those in other proteins containing non-heme, non-sulfur diiron sites [2]. Therefore, the location of the diiron site ligands in Rr is not so obvious.

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In this communication we present evidence for internal sequence homology within the Rr subunit. The two homologous regions each contain a conserved Glu and His residue which, by comparison with similar regions in other proteins, could provide ligands to the diiron site.

MATERIALS AND METHODS

Materials: Chemicals and biochemicals were from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) except where noted. Sodium dithionite was from BDH Chemicals Ltd., Poole, UK. Partially purified Rr from *Desulfovibrio vulgaris* (Hildenborough) was generously provided by Professor Jean LeGall and purified to homogeneity as previously described [1].

Sequence analyses: The amino acid sequence of rubrerythrin was derived from the nucleotide sequence, as described in [2]. Other sequences were obtained from the Protein Identification Resource (PIR) databank (*P. gouldii* hemerythrin [3], uteroferrin [4], B2 subunit of *E. coli* ribonucleotide reductase [5,6], and Component A α subunit of *Methylosinus capsulatus* methane monooxygenase [7]), or from the original literature (Component A α subunit of *M. trichosporium* methane monooxygenase [8]). Amino acid sequence comparisons were performed using the Genetics Computer Group software package [9] to generate comparisons with the COMPARE and BESTFIT programs. Default parameters were used in all cases, except that a stringency value of 17 was used in the COMPARE analysis. The stringency score for any two compared sequences represents the sum of identities (1.0) plus conservative substitutions ($\geq$ 0.5) according to the Dayhoff comparison matrix.

Activity Assays: Inorganic pyrophosphatase activity of Rr was determined at 37 °C by colorimetric determination of inorganic phosphate released from sodium pyrophosphate [10] or at 25 °C by the method of Taussky and Schorr [11]. Assays were performed under both aerobic and reducing conditions. Assays under reducing conditions were performed under an Ar atmosphere and included 100 μM sodium dithionite in the reaction mixture. Yeast inorganic pyrophosphatase (Sigma Chemical Co.) yielded a specific activity in the aerobic assay which was close to that cited by the manufacturer.

Assays of Rr for methane monooxygenase activity were performed under single turnover conditions as described previously for methane monooxygenase component A [12]. All stock solutions were degassed and placed under an Ar atmosphere prior to mixing. Solutions were transferred via gas-tight syringes. A 100 μL aliquot of a stock solution of reaction mixture (10 μM proflavin and 100 μM methyl viologen in 0.1 M Tris-HCl at pH 7.6) was transferred to an anaerobic 3 ml septum-capped vial. A solution of Rr (1 to 10 nmoles) was injected into the reaction vial and reduced with two equivalents of sodium dithionite, giving a total reaction volume of ~100 μl. The reaction was incubated for 5 min at 30 °C. Propene gas (1 ml) was then injected and the mixture was incubated at 30 °C for a further 5-10 min. The reaction was initiated by addition of 1 ml of air or 1 μmole of H_2O_2 (from a 100 mM stock solution). The reaction was stopped by addition of 100 μL of CCl_4 at times ranging from 5 minutes to twelve hours following addition of O_2 or H_2O_2. The resulting mixture was then analyzed for the presence of propene oxide by gas chromatography on a Varian Vista 6000 chromatograph using a Porapak Q column.

RESULTS AND DISCUSSION

Identification of homologous regions in the Rr peptide and potential diiron site ligand residues: The results of an internal homology search on the amino acid sequence of the 21.5

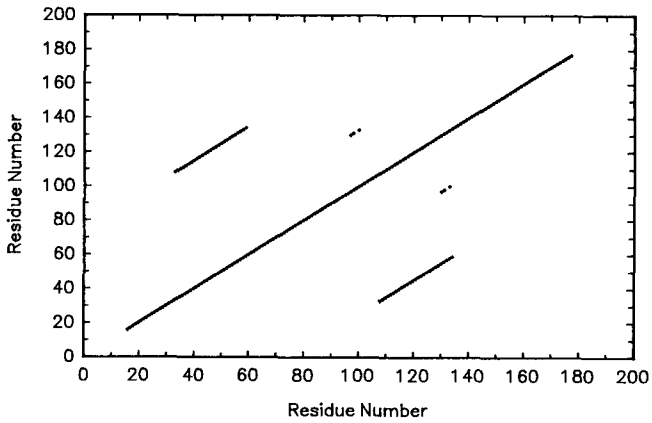

Figure 1. Amino acid sequence homology comparison of Rr vs Rr using the Genetics Computer Group program, COMPARE. Points were placed at the midpoints of 30-residue comparison regions ("sequence windows") along the two axes whenever a stringency score of 17 was met or exceeded.

kDa subunit of Rr using the COMPARE program is illustrated in Fig. 1. The two longer diagonal lines flanking the central diagonal depict two non-identical regions of the Rr polypeptide which share a high degree of sequence homology. These two highly homologous regions, labelled Rr1 and Rr2, are aligned with each other in Fig. 2 according to the BESTFIT program. Residues 30 through 63 (Rr1) and 105 through 138 (Rr2) contain 12 identical residues and 8 conservative substitutions. Rr1 and Rr2 lie outside the C-terminal portion of the Rr sequence (residues 153-191) which was previously identified as homologous to rubredoxin sequences [2]. The Rr1 and Rr2 sequences contain a homologous pair of His and Glu residues (indicated in bold in Fig. 2). Since His and Glu residues are known to

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      30                                                  63
Rr1:  FGGQAKKDGFVQISDIFAETADQEREHAKRLFKF
      |:  |: :|: :|  :||  |  |  |  ||:: |
Rr2:  FARIAREEGYEEIARVFASIAVAEEFHEKRFLDF
     105       | |  : ||  ||:  |  |       138
MMO:           GDEITPTVFLSIETDELRH
              228                 246
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Figure 2. Sequence alignment of the two 34-residue regions of homology, Rr1 and Rr2, which are depicted diagrammatically in Fig. 1. A portion of the sequence of component A α subunit of *M. capsulatus* methane monooxygenase (MMO) is aligned with Rr2. Between each pair, the vertical lines indicate identical residues, and double dots indicate conservative substitutions. Residue numbers are given above or below the respective sequences. The conserved Glu and His residues are in bold, and the underlines indicate conserved EXXH sequences.

furnish iron ligands in proteins containing non-heme, non-sulfur diiron sites, this observation prompted us to undertake sequence comparisons between Rr1 or Rr2 and each of these other proteins. Using the BESTFIT program, no significant homology was found between Rr1 or Rr2 and any stretch of the hemerythrin, uteroferrin or ribonucleotide reductase B2 subunit sequences. However, significant homology *was* found between residues 228-246 of the component A α subunit of methane monooxygenase and Rr2 (cf. Fig. 2). This latter homology is noteworthy because the aligned Glu and His residues in the methane monooxygenase sequence are one of a pair which were suggested to be iron ligands [8]. This suggestion was based on homology to a pair of EXXH sequences which are known to provide Glu and His ligands to each iron in the ribonucleotide reductase B2 subunit [5]. As can be seen from Fig. 2, a pair of EXXH sequences occurs in the internally homologous regions of the Rr sequence as well. Analysis of the Rr sequence using the algorithm of Chou and Fasman [13] indicates that both the Rr1 and Rr2 sequences occur within predominantly α-helical regions, which is reminiscent of the secondary structure surrounding the diiron sites in hemerythrin [14,15] and ribonucleotide reductase [5]. Taken together, these observations suggest that the conserved Glu and His residues at positions 53, 56, 128, and 131 in the Rr sequence are reasonable candidates as ligands to the diiron site. The published stoichiometry indicates one diiron site iron atom per 21.5 kD subunit of Rr [1], whereas the preceding discussion implies two such iron atoms per subunit. This observation is not unlike the history of the iron stoichiometry for ribonucleotide reductase, which was initially reported to be one iron atom per B2 subunit, but has more recently been shown to be two [5].

Activity assays of Rr: Because of the apparent sequence homology between methane monooxygenase and Rr shown in Figure 2, we tested the activity of Rr toward oxidation of propene to propene oxide under single turnover conditions. In the case of methane monooxygenase, this assay allows determination of the activity of the iron-containing component A in the absence of other protein components. These assays were carried out on Rr whose iron analyses indicated one diiron site iron per rubredoxin-like iron, which is the same as the published ratio [1]. No production of propene oxide from propene was detected using either O_2 or H_2O_2 as oxidant, suggesting that Rr by itself is not competent to catalyze this conversion. The absence of any apparent sequence homology between Rr and uteroferrin (whose non-heme, non-sulfur diiron site functions as a phosphatase [16]) prompted us to reinvestigate the recently reported pyrophosphatase activity of Rr [17]. Our measured specific activity of Rr in the aerobic assay at 37 °C was 0.003 μmole min^{-1} mg^{-1}, which is < 1/1000 of that reported in [17]. No pyrophosphatase activity of Rr was detected at 25 °C or under reducing conditions. Thus, the physiological function of Rr remains

unclear. Nevertheless, our results indicate that a pair of EXXH sequences represents a diiron binding motif in Rr, as in ribonucleotide reductase and methane monooxygenase.

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