Catalysis in fumarate reductase

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Abstract

In the absence of oxygen many bacteria are able to utilise fumarate as a terminal oxidant for respiration. In most known organisms the fumarate reductases are membrane-bound iron-sulfur flavoproteins but *Shewanella* species produce a soluble, periplasmic flavocytochrome c_3 that catalyses this reaction. The active sites of all fumarate reductases are clearly conserved at the structural level, indicating a common mechanism. The structures of fumarate reductases from two *Shewanella* species have been determined. Fumarate, succinate and a partially hydrated fumarate ligand are found in equivalent locations in different crystals, tightly bound in the active site and close to N5 of the FAD cofactor, allowing identification of amino acid residues that are involved in substrate binding and catalysis. Conversion of fumarate to succinate requires hydride transfer from FAD and protonation by an active site acid. The identity of the proton donor has been open to question but we have used structural considerations to suggest that this function is provided by an arginine side chain. We have confirmed this experimentally by analysing the effects of site-directed mutations on enzyme activity. Substitutions of Arg402 lead to a dramatic loss of activity whereas neither of the two active site histidine residues is required for catalysis. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Shewanella species are widespread Gram-negative proteobacteria and are particularly abundant in marine and freshwater sediments. They are remarkable in their diversity of respiratory pathways [1,2] and can utilise many inorganic and organic electron acceptors, including fumarate, nitrate, trimethylamine *N*-oxide, thiosulphate and, more unusually, insoluble oxides of Fe(III) and Mn(IV). The diversity of known respiratory pathways is matched by a large number of redox proteins. Several have been identified biochemically but it is clear from examination of the genome sequence of *Shewanella* MR-1 (available at www.tigr.org) that a very large number of uncharacterised cytochromes and other electron transfer proteins are produced. When *Shewanella frigidimarina* NCIMB400 (formerly *Shewanella putrefaciens*) is grown anaerobically, it produces large quantities of several *c*-type cytochromes [3]. The most abundant of these is a 64 kDa flavocytochrome that catalyses methyl viologen-dependent fumarate reduction in vitro [4,5]. The role of this flavocytochrome c_3 (Fcc$_3$) in fumarate respiration has been shown using a null

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mutant constructed by gene disruption [6]. The mutant is incapable of fumarate respiration but pathways to other electron acceptors are unaffected. This specific defect shows not only that flavocytochrome c_3 is required for fumarate respiration but its function cannot be performed by other proteins produced under these conditions. This implies that no membrane-bound fumarate reductase is produced. It appears, surprisingly, that the genome of *Shewanella* MR-1 includes an operon that is very similar to the *Wolinella Frd* operon that encodes the membrane-bound fumarate reductase, even though this strain also produces a soluble flavocytochrome c_3 fumarate reductase [7]. Whether a similar *Frd* operon is present in NCIMB400 is not yet known. If so then its role in fumarate respiration is unclear since the Fcc$_3$ knockout strain failed to grow with fumarate as terminal electron acceptor. It has been shown that *S. frigidimarina* NCIMB400 produces a second flavocytochrome c_3 (Ifc$_3$) which also efficiently reduces fumarate in vitro [8]. However, this protein is specifically induced by Fe(III) and its physiological function is poorly understood.

Shewanella species are the only bacteria known to produce soluble respiratory fumarate reductases. In other organisms fumarate is reduced by a membrane-bound complex of either three or four subunits [9]. These enzymes are closely related in structure and activity to succinate dehydrogenases which catalyse the reverse reaction. All fumarate reductases and succinate dehydrogenases contain FAD at the active site but electron transfer to the flavin is mediated by three iron-sulfur centres in the membrane-bound enzymes whereas the four haem centres of flavocytochrome c_3 perform the equivalent function. The FAD-binding catalytic domain or subunit is highly conserved within and between these groups of enzymes indicating that the catalytic mechanism is likely to be very similar in all cases.

2. Fumarate reductase structure

The determination of the high-resolution crystal structure of *Shewanella* fumarate reductase ([10,11]; PDB references 1QJD, 1D4C) has provided major insights into substrate binding and catalysis. The protein consists of a single polypeptide organised into three distinct domains (Fig. 1). The small, N-terminal, cytochrome domain containing four haem groups is tethered to the flavin domain by a charged bent helix linker (residues 100–110). The flavin do-
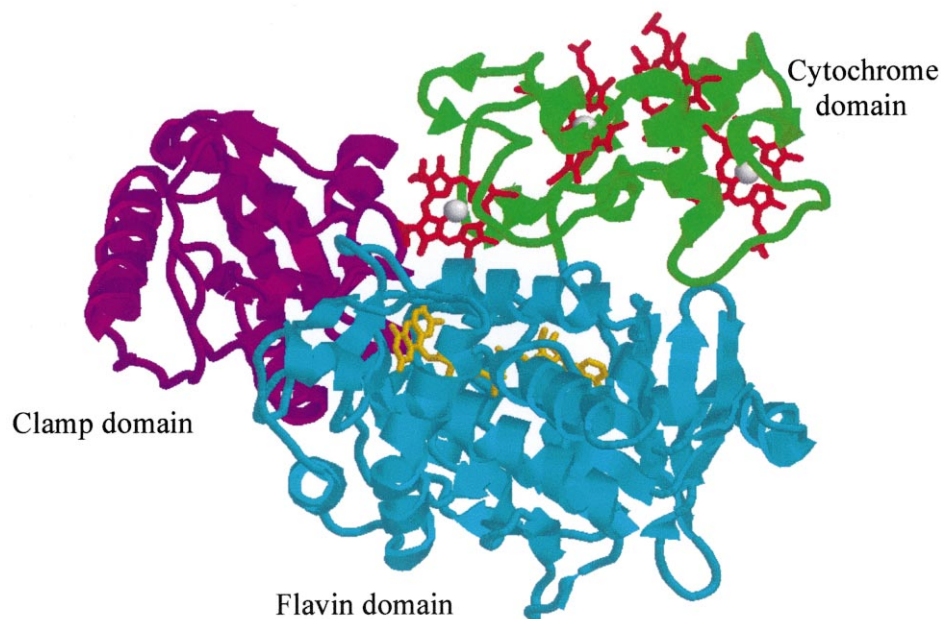

Fig. 1. Domain structure of *S. frigidimarina* fumarate reductase. The polypeptide chain is shown as a ribbon diagram with the cytochrome, flavin-binding and clamp domains coloured green, cyan and magenta, respectively. The haem groups are shown in red (with the iron in grey) and the flavin is in yellow.

main (residues 111–364 and 503–571, coloured cyan in Fig. 1) holds the non-covalently bound FAD group. The overall fold of the FAD binding domain has a structural and topological similarity with known FAD binding proteins, though sequence similarity is poor [12]. Buried within the flavin domain is an octahedrally coordinated sodium ion which is close to the active site and may well play a structural or regulatory role. The clamp domain of Fcc$_3$ (residues 365–502) is likely to be involved in controlling access of substrate to the active site. It consists of seven short helical stretches wrapped round a four-stranded antiparallel sheet.

The crystallisation medium contained a 10 mM solution of fumarate, well above the K_m of 25 μM, suggesting that the active site of Fcc$_3$ should be fully occupied. However, the electron density map clearly shows the presence of a hydrated, malate-like molecule in the active site of Fcc$_3$ (Fig. 2). It is unlikely that this molecule is oxaloacetate because the protein is crystallised from fully active enzyme in the complete absence of this compound. The malate-like molecule is held tightly by several hydrogen bonds and sits in a close-fitting binding site that is completely inaccessible to solvent [10].

The absence of a solvent-accessible channel to the active site implies that there must be significant domain movement to enable substrate binding and product release. The active site is at the interface between the flavin-binding and clamp domains, both of which contribute important active site residues. We presume that the closed conformation that we observe when crystals are grown in the presence of fumarate represents a close approximation to the catalytically competent state. The enzyme is found in this form with either substrate or product bound in the active site [10] and it also appears that, in the absence of available reducing equivalents, the enzyme is still capable of modifying the substrate by nucleophilic attack [11]. The relative positions of the two domains are somewhat variable in the different fumarate reductase structures and more especially in L-aspartate oxidase [13], a closely related enzyme that can also reduce fumarate. This protein was crystallised in an open conformation with no substrate bound – indeed the FAD cofactor had also dissociated. We conclude that domain movement is an important feature of these enzymes with
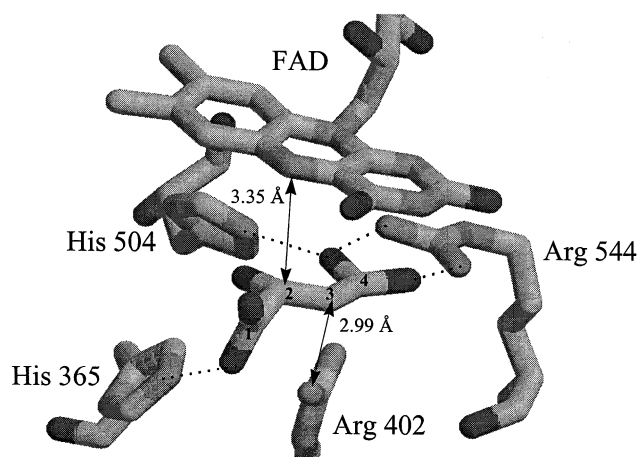

Fig. 2. The environment of the modified substrate in the active site. The malate-like molecule is shown with hydrogen bonds to Arg544, His504 and His 365 as dotted lines. The distances relevant for hydride and proton transfer are indicated.

opening being necessary for access to the active site and closure being essential for catalysis as described below.

3. Electron transfer

The pathway from membrane-bound quinols to the fumarate reductase in *Shewanella* is not well understood, but a membrane-bound tetrahaem cytochrome *c* has been implicated in electron transfer to fumarate reductase and other terminal reductases. This protein, CymA [14], is related to the NirT/NapC family. It is not known whether this protein directly reduces flavocytochrome c_3 but the organisation of the Fcc$_3$ cytochrome domain, with at least three of its four haems highly accessible to solvent, indicates that electron transfer should be facile. The edge-to-edge distances between pairs of haems in Fcc$_3$ range from a mere 3.9 Å to 8.0 Å and the shortest haem-FAD distance is just 7.4 Å. All of these distances are commensurate with very rapid internal electron transfer. The thermodynamic properties of the redox centres have been determined by potentiometric measurements and by protein film voltammetry [15]. The reduction potentials of the haems are low, ranging from −238 to −102 mV at pH 7.0 and 25°C with the FAD potential at −154 mV, giving a substantial driving force for fumarate reduction.

4. Substrate binding and catalysis

The malate-like molecule found in the active site is tightly bound and inaccessible to solvent. Its binding is indistinguishable from fumarate bound in the active site of fumarate reductase from *Shewanella* MR-1 [11]. The C4 carboxylate is bound in a highly polar environment by electrostatic interaction and hydrogen bonds to Arg544 and Arg402 and by a hydrogen bond to His504 (Fig. 2). The environment of the C1 carboxylate is much less polar but hydrogen bond interactions with Thr377 and His365 are observed.

Two significant features of substrate binding appear critical for the reaction mechanism (Fig. 3). Normally fumarate is a symmetrical, planar, substrate. Reduction to succinate involves the transfer of a hydride from N5 of the FAD to C2 and a proton to C3 resulting in a non-planar product. This reaction is facilitated by an induced polarisation of the substrate, resulting from the charge asymmetry in the binding pocket and by an induced loss of planarity upon binding to the enzyme. This results from the close interaction of the C1 carboxylate with the side chains of Met236 and Met375, too close to allow substrate to bind as a planar molecule. Met236 is in the flavin-binding domain whereas Met375 is a clamp domain residue so it seems that the substrate distortion is a feature of domain closure around the active site. The orientation of the C1 carboxylate by hydrogen bonds to His365 and Thr377 may facilitate domain closure and provide the drive to twist the substrate out of the planar conformation with the additional effect of weakening the conjugated double bonds. The exceptionally polar hydrogen bonding environment of the C4 carboxyl group with contributions from two arginines and a histidine, acts to polarise the fumarate. The combined steric and electronic effects then reinforce each other to generate considerable positive charge at C2 making it amenable to nucleophilic attack.

The flavin N5 is positioned less than 3.2 Å from fumarate C2, poised to attack the si-face of the C2 centre. Hydride transfer to C2 is followed by protonation of C3, resulting in formation of succinate; the only residue sufficiently close to C3 for protonation is Arg402 at a distance of 2.99 Å. The role of this residue is discussed in detail below. Under the oxi-
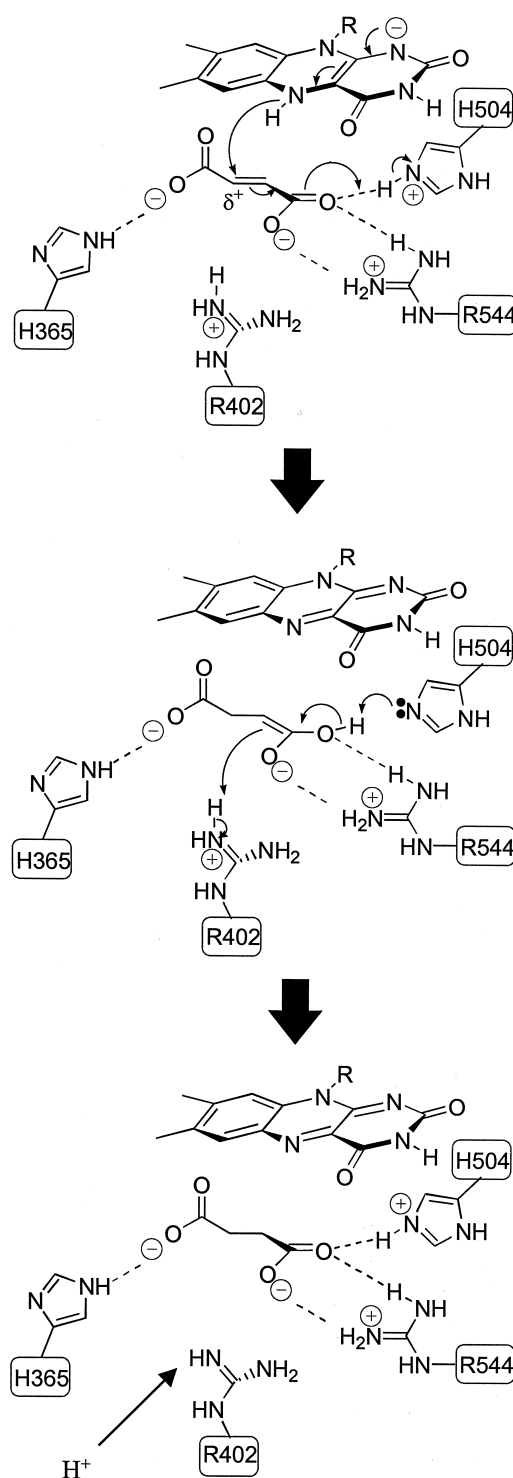

Fig. 3. The mechanism of fumarate reduction. The key residues are shown schematically with hydrogen bonds as dashed lines. The proposed mechanism is described in the text.

dising conditions that were used for crystal growth, hydride attack is not possible and instead there is attack by H_2O on the re-face to provide the observed product which has *R*-stereochemistry at C2 [10].

5. Proton transfer

The structures of five fumarate reductases from *Shewanella* [10,11,16], *Escherichia coli* [17] and *Wolinella succinogenes* [18] indicate a common mechanism for substrate binding and activation, suitably positioned for hydride transfer. Completion of product formation also requires protonation at C3 and the source of the proton has been the subject of some debate.

Prior to the crystallographic results, it was generally expected that a histidine would act as the acid/base catalyst in fumarate reductases and succinate dehydrogenases. This expectation was based on the widespread use of histidines in proton transfer in many types of enzyme and the fact that chemical modification studies with succinate dehydrogenase indicated that a histidine residue played an important catalytic role [19]. Analysis of the structure shows that the two active site histidines, His365 and His504, are each hydrogen-bonded to substrate carboxylate oxygens and not well positioned for proton transfer, being too distant from the substrate C3. To probe the roles of these residues and their possible involvement in substrate protonation as suggested elsewhere (e.g. [16]) we have used site-directed mutagenesis to convert each to alanine [20]. The H365A and H504A mutant enzymes exhibited lower k_{cat} values than the wild-type enzyme (Table 1) but only by factors of 3–15, depending on pH. This, coupled with the increase in K_m observed for these enzymes, indicates that His365 and His504 are primarily involved
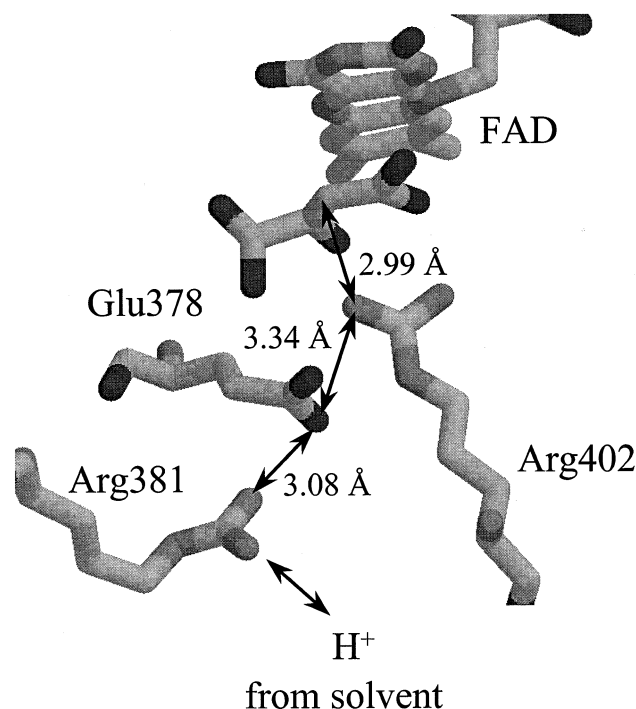

Fig. 4. The putative proton delivery pathway. Arg381 is exposed to solvent and close interactions indicate a pathway for rapid proton transfer to the substrate via Glu378 and Arg402.

in Michaelis complex formation and are not essential catalytic residues.

The structures of two of the *Shewanella* enzymes [10,11] and *E. coli* fumarate reductase [17] clearly show NH2 of Arg402 close to the substrate C3, the eventual proton acceptor. When we altered Arg402 to Ala we could not detect fumarate reductase assay in the recombinant enzyme, indicating a key role for this arginine. Interestingly we have recently substituted Arg402 with a lysine residue and the enzyme now exhibits activity, albeit at an extremely low level (Table 1).

Since the active site is inaccessible to solvent, delivery of a proton to Arg402 may appear problematic. However, a putative pathway for proton transfer is readily observed in that Glu378 forms a bridge between Arg402 and a conserved surface residue, Arg381 (Fig. 4). These residues are completely conserved, consistent with such an important function.

In the structure of the *Wolinella* fumarate reductase [18], the position of the residue equivalent to Arg402 in the *Shewanella* enzyme (Arg301) has moved by about 3 Å compared to the *Shewanella*

Table 1
Comparison of k_{cat} and K_m values for wild-type, H365A, H504A and R402K Fcc3 (pH 7.2, 25°C, $I = 0.45$ M)

Form of Fcc3	k_{cat} (s^{-1})	K_m (μM)
Wild-type	509 ± 15	25 ± 10
H365A	51 ± 2	259 ± 24
H504A	65 ± 3	256 ± 23
R402K	0.055 ± 0.004	66 ± 14

No activity was detected with the R402A enzyme.

and *E. coli* enzyme structures. An overlay of the flavin binding domains of all structures shows that the conserved catalytic residues of the flavin-binding domain are very similar. The residues on the clamp domain differ in position by between 1 and 3 Å, corresponding to an opening of the clamp in the *Wolinella* enzyme structure [18]. It should be noted that this structure was determined with crystals that had had substrate diffused in – they may have been trapped in a slightly open conformation. The fact that the position of the arginine side chain is somewhat altered in the *Wolinella* fumarate reductase has led Lancaster and colleagues to propose that the proton comes instead from a water molecule [18]. However, if the clamp domain in the *Wolinella* enzyme is moved to the orientation (i.e. the closed form) seen in the other fumarate reductases then the position of the arginine side chain is comparable to that seen in all the other structures. Thus we are convinced that our mechanism (Fig. 3), involving hydride transfer from flavin N5 and proton transfer from this conserved arginine residue, applies for all members of the fumarate reductase family.

Acknowledgements

References

[1] C.R. Myers, K.H. Nealson, Science 240 (1988) 1319–1321.

[2] K.H. Nealson, D.A. Saffarini, Annu. Rev. Microbiol. 48 (1994) 311–343.

[3] C.J. Morris, D.M. Gibson, F.B. Ward, FEMS Microbiol. Lett. 69 (1990) 259–262.

[4] S.L. Pealing, A.C. Black, F.D.C. Manson, F.B. Ward, S.K. Chapman, G.A. Reid, Biochemistry 31 (1992) 12132–12140.

[5] S.L. Pealing, M.R. Cheesman, G.A. Reid, A.J. Thomson, F.B. Ward, S.K. Chapman, Biochemistry 34 (1995) 6153–6158.

[6] E.H.J. Gordon, S.L. Pealing, S.K. Chapman, F.B. Ward, G.A. Reid, Microbiology 4 (1998) 937–945.

[7] C.R. Myers, J.M. Myers, FEMS Microbiol. Lett. 98 (1992) 13–19.

[8] P.S. Dobbin, J.N. Butt, A.K. Powell, G.A. Reid, D.J. Richardson, Biochem. J. 342 (1999) 439–448.

[9] T. Ohnishi, C.C. Moser, C.C. Page, P.L. Dutton, T. Yano, Structure 8 (2000) R23–R32.

[10] P. Taylor, S.L. Pealing, G.A. Reid, S.K. Chapman, M.D. Walkinshaw, Nat. Struct. Biol. 6 (1999) 1108–1112.

[11] D. Leys, A.S. Tsapin, K.H. Nealson, T.E. Meyer, M.A. Cusanovich, J.J. van Beeumen, Nat. Struct. Biol. 6 (1999) 1113–1117.

[12] M.W. Fraaije, A. Mattevi, Trends Biochem. Sci. 25 (2000) 126–132.

[13] A. Mattevi, G. Tedeschi, L. Bacchella, A. Coda, A. Negri, S. Ronchi, Structure 7 (1999) 745–756.

[14] C.R. Myers, J.M. Myers, J. Bacteriol. 179 (1997) 1143–1152.

[15] K.L. Turner, M.K. Doherty, H.A. Heering, F.A. Armstrong, G.A. Reid, S.K. Chapman, Biochemistry 38 (1999) 3302–3309.

[16] V. Bamford, P.S. Dobbin, D.J. Richardson, A.M. Hemmings, Nat. Struct. Biol. 6 (1999) 1104–1107.

[17] T.M. Iverson, C. Luna-Chavez, G. Cecchini, D.C. Rees, Science 284 (1999) 1961–1966.

[18] C.D. Lancaster, A. Kröger, M. Auer, H. Michel, Nature 402 (1999) 377–385.

[19] A.D. Vinogradov, Biokhimiya 51 (1986) 1944–1973.

[20] M.K. Doherty, S.L. Pealing, C.S. Miles, R. Moysey, P. Taylor, M.D. Walkinshaw, G.A. Reid, S.K. Chapman, Biochemistry (2000) in press.