A Proton Delivery Pathway in the Soluble Fumarate Reductase from *Shewanella frigidimarina*†

Received for publication, March 31, 2006, and in revised form, May 4, 2006 Published, JBC Papers in Press, May 12, 2006, DOI 10.1074/jbc.M603077200

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The mechanism for fumarate reduction by the soluble fumarate reductase from *Shewanella frigidimarina* involves hydride transfer from FAD and proton transfer from the active-site acid, Arg-402. It has been proposed that Arg-402 forms part of a proton transfer pathway that also involves Glu-378 and Arg-381 but, unusually, does not involve any bound water molecules. To gain further insight into the importance of this proton pathway we have perturbed it by substituting Arg-381 by lysine and methionine and Glu-378 by aspartate. Although all the mutant enzymes retain measurable activities, there are orders-of-magnitude decreases in their *k*<sub>cat</sub> values compared with the wild-type enzyme. Solvent kinetic isotope effects show that proton transfer is rate-limiting in the wild-type and mutant enzymes. Proton inventories indicate that the proton pathway involves multiple exchangeable groups. Fast scan protein–film voltammetric studies on wild-type and R381K enzymes show that the proton transfer pathway delivers one proton per catalytic cycle and is not required for transporting the other proton, which transfers as a hydride from the reduced, protonated FAD. The crystal structures of E378D and R381M mutant enzymes have been determined to 1.7 and 2.1 Å resolution, respectively. They allow an examination of the structural changes that disturb proton transport. Taken together, the results indicate that Arg-381, Glu-378, and Arg-402 form a proton pathway that is completely conserved throughout the fumarate reductase/succinate dehydrogenase family of enzymes.

Proton transfer and its coupling to catalytic electron transfer in proteins is an integral feature of bioenergetics and is fundamental to the conservation of biological energy. Proton transfer is widely considered to involve a chain of closely spaced donors and acceptors, either water molecules or amino acid side chains, and it is the generally held view that water molecules are an essential feature in the movement of protons via a Grotthuss mechanism (1). The conduction of the proton along the chain consists of translocation of an excess proton (propagation of an ionic defect) followed by reorientation of the hydrogen-bonded chain (propagation of a bonding defect). Hence, the assumption of Grotthuss-like behavior is a common starting point in biomolecular proton transfer, allowing the proton to be conducted through the protein matrix. There are numerous examples of proton transfer pathways within proteins. Perhaps the most intensely studied have been the photosynthetic reaction center from *Rhodobacter sphaeroides* (2), cytochrome *c* oxidase (3, 4), carbonic anhydrase (5), the membrane-spanning light-driven proton pump bacteriorhodopsin, synthesized by many Haloarchaea (6), and the 7Fe ferredoxin from *Azotobacter vinealandii* (7). With the exception of the latter case, in which electrochemical kinetics and structures at atomic resolution lead to a mechanism involving a single “swinging arm” carboxylate, all the proton transfer pathways studied to date include at least one water molecule.

Flavocytochrome *c*<sub>3</sub>, isolated from the marine bacterium *Shewanella frigidimarina*, is a tetraheme FAD-containing flavoenzyme designated as Fcc<sub>3</sub>.<sup>5</sup> Produced in the absence of oxygen as a soluble enzyme in the bacterial periplasm, it allows the bacterium to respire using fumarate as the terminal electron acceptor. Although a number of other bacteria also express fumarate reductases, these are generally found as membrane-bound multisubunit enzymes containing soluble iron-sulfur and FAD-containing domains and are anchored to the inner face of the cytoplasmic membrane by either one or two predominantly α-helical subunits (8, 9). The crystal structures of a number of members of the fumarate reductase/succinate dehydrogenase family have been solved including the membrane-bound complexes from *Escherichia coli* (PDB codes 1L0V (8) and 1NEK (10)) and *Wolinella succinogenes* (PDB entries 1QLA, 1QLB (11), and 1E7P (12)) and the soluble fumarate receptor.

**References**

1. Supported by studentship funding from the Engineering and Physical Sciences Research Council.
2. Supported by St. Edmund Hall, University of Oxford Clarendon Fund and University of Sydney Travelling Scholarship.
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5. The abbreviations used are: Fcc<sub>3</sub>, flavocytochrome *c*<sub>3</sub>; P.L., the equivalent of pH in mixed isotope solvents; SKIE, solvent kinetic isotope effect; WT, wild type; MES, 2-morpholinoethanesulfonic acid; CHES, 2-(cyclohexylamino)-ethanesulfonic acid; TAPS, N-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

*This work was supported in part by the United Kingdom Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

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§§‡ The atomic coordinates and structure factors (code 2B7R (E378D) and 2B7S (R381K)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

*‡* Received for publication, March 31, 2006, and in revised form, May 4, 2006. Published, JBC Papers in Press, May 12, 2006, DOI 10.1074/jbc.M603077200.
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reductases from the *Shewanella* family (PDB codes 1QJD (13), 1D4E (14), and 1QO8 (15)). Examination of the available crystal structures shows conservation of the active site architecture, consistent with a universal mechanism of fumarate reduction (13, 16–18).

On the basis of the highest resolution structure, the 1.8-Å structure of the *S. frigidimarina* Fcc₃ enzyme, a mechanism was proposed that involves an arginine (Arg-402) donating a proton to the substrate during catalysis. This residue is thought to be reprotolated via a proton pathway consisting of two further residues (Glu-378 and Arg-381) (16, 17, 19), also found to be completely conserved throughout the fumarate reductase/sucindate dehydrogenase family. The proposed proton pathway in flavocytochrome *c₃* involves the proton moving from the guanidinium group of Arg-381 to the carboxylate group of Glu-378 before finally reaching the guanidinium group of the active-site acid, Arg-402. The structure of the proton delivery pathway and the juxtaposition of these residues to the active site is shown in Fig. 1. Unusually, this proton transfer pathway does not involve any bound water molecules.

To gain further insight into proton delivery to the active site in this soluble fumarate reductase, we have altered the structure of the proton pathway by substituting Arg-381 with lysine and methionine and Glu-378 with aspartate. Here we report the kinetic characterization of these mutants in addition to the crystal structures of the E378D and R381K mutant enzymes to 1.7 and 2.1 Å resolution, respectively.

MATERIALS AND METHODS

**DNA Manipulation, Strains, Media, and Growth**—The mutant enzymes E378D, R381K, and R381M were generated by site-directed mutagenesis using the method of Kunkel and Roberts (20) as described previously (16). The mutagenic oligonucleotides 5'-GGTCACGTATGCAGGTACGTG (which substitute glutamic acid 378 by aspartic acid), 5'-GAAGCGGTAAGGTATGTTG, and GAAGCGGTAATGGTG (which substitute arginine 381 with lysine and methionine, respectively) were used. Mismatched bases are underlined. After complete sequencing, the modified coding sequences were cloned into the broad-host range expression plasmid pMMB503EH (21) on an ~1.8-kbp EcoRI-HindIII fragment to generate pCM100 (E378D fecA/pMMB503EH), pCM17 (R381K fecA/pMMB503EH), and pCM19 (R381M fecA/pMMB503EH). Expression of the mutant enzymes in the ΔfecA *S. frigidimarina* strain EG301 (22) was carried out as described previously (16).

**Protein Purification and FAD Determination**—Wild-type and mutant forms of Fcc₃ were purified as previously reported (23). Protein samples for crystallization, mass spectrometry, and protein film voltammetry were subjected to an additional purification step using fast protein liquid chromatography with a 1-ml Resource Q column (GE Healthcare) as described by Pealing et al. (24). Protein concentrations were determined using the Soret band absorption coefficient for the reduced enzyme (752.8 mm⁻¹ cm⁻¹ at 419 nm) (24). The FAD content of Fcc₃ mutants was determined using the method of Macheraux (25), and all steady-state rate constants were corrected for the percentage of FAD present.

Mass spectrometry of proteins was carried out using a Micromass Platform II Electrospray mass spectrometer. Samples were prepared in 0.1% (v/v) formic acid before being diluted 1:1 with acetonitrile and introduced to the spectrometer via direct infusion. The spectrometer was standardized using horse heart myoglobin.

**Steady-state Kinetics**—The steady-state solution kinetics of fumarate reduction were measured at 25 °C as described by Turner et al. (26). The fumarate-dependent reoxidation of reduced methyl viologen was monitored at 600 nm using a Shimadzu UV-PC 1501 spectrophotometer. To ensure anaerobicity, the spectrophotometer was housed in a Belle Technology glove box under a nitrogen atmosphere with the O₂ level maintained below 2 ppm. Assay buffers contained 0.45 M NaCl and 0.2 mM methyl viologen and were adjusted to the appropriate pH values using 0.05 M HCl or NaOH as follows: Tris/HCl (pH 7.0–9.0); MES/NaOH (pH 5.4–6.8); CHES/NaOH (pH 8.6–10). The viologen was reduced by the addition of sodium dithionite until a reading of ~1 absorbance unit was obtained (corresponding to ~80 µM reduced methyl viologen). The concentration of reduced methyl viologen could be varied between 100 and 20 µM with no effect on the rate of reaction. A known concentration of enzyme was added, and the reaction was initiated by the addition of fumarate (0–1 mM). Kinetic parameters *k*ₘ and *k*ₐ were determined by nonlinear regression analysis (Microcal Origin Software).

**Solvent Kinetic Isotope Effects (SKIE)**—The effect of solvent deuteration on turnover rates was fol-
lowered at 25 °C as described by Rothery et al. (27) via the steady-state assay method detailed above. Buffer and substrate solutions were prepared in both D$_2$O and H$_2$O, and assays were performed under saturating fumarate conditions. The pH was adjusted by titrating DCl or NaOD into the deuterated buffer, applying the equation pD = pH meter reading + 0.4 to correct for the acidity of the pH electrode itself (28). The stock protein solution was prepared in H$_2$O and then concentrated such that the addition of the protonated enzyme solution was less than 0.05% of the total volume of the assay. The percentage of D$_2$O in the addition of the protonated enzyme solution was less than 0.05% of the total volume of the assay. The percentage of D$_2$O in the buffer was varied between 0 and 100% by adding the appropriate proportions of deuterated and protonated buffer in the assay cuvette. The protein was allowed to equilibrate with the deuterated buffer for ~30 s before initiation of the assay by the addition of fumarate. The rate constants were plotted as a function of the D$_2$O concentration to construct a thermal measurement, and electrode potentials were conducted in an anaerobic glove box (Vacuum Atmospheres, O$_2$ < 2 ppm). The all-glass electrochemical cell (30) was contained within a Faraday cage (1-cm mesh) to decrease electrical noise. The saturated calomel electrode (SCE) was located in a well separated side arm maintained at 25 °C (to allow non-isothermal measurement), and electrode potentials were converted to the standard hydrogen electrode scale using the relationship E$_{SHE}$ = E$_{SCE}$ + 0.241 V at 25 °C (31). The pyrolytic graphite electrode rotating disc electrode (~3-mm$^2$ surface area, constructed as described previously) was polished with 1-μm alumina (Buehler). Each film of Fcc$_3$ was formed by adsorption at 5 °C from a dilute solution (1 ml of buffer with 0.4 mM enzyme) containing 200 mg ml$^{-1}$ polymyxin as co-adsorbate to stabilize the enzyme on the electrode. The electrode was inserted into the cell solution, and the potential was cycled to stabilize the enzyme film on the electrode. The electrode surface.

**Protein Film Voltammetry**—Voltammetric experiments were conducted in a 3-mm$^2$ surface area, constructed as described previously) was polished with 1-μm alumina (Buehler). Each film of Fcc$_3$ was formed by adsorption at 5 °C from a dilute solution (1 ml of buffer with 0.4 mM enzyme) containing 200 mg ml$^{-1}$ polymyxin as co-adsorbate to stabilize the enzyme on the electrode. The electrode was inserted into the cell solution, and the potential was cycled twice between 0.24 and −0.65 V at 0.02 V s$^{-1}$, whereas it was rotated at 200 rpm. After a stable film had formed, as judged from the appearance of well defined signals (usually 4–6 cycles), the enzyme solution was replaced by a clean buffer solution containing all components except enzyme, and the temperature was adjusted to the required value. Voltammograms were recorded using an Autolab system (EcoChemie, Utrecht, The Netherlands) equipped with SCANGEN and ADC750 (analogue-digital converter) modules for fast scan rates (>1 V s$^{-1}$) and an ECD (electrochemical detection) module to improve current sensitivity at slower scan rates (< 0.1 V s$^{-1}$). Uncompensated resistance was minimized using the positive-feedback IR (solution resistance) compensation feature. For electrocatalytic measurements the electrode was rotated using an EG&G M636 electrode rotator (Princeton Applied Research) to control mass transport of fumarate to the electrode surface.

**Crystallization and Refinement**—Crystallization of E378D and R381K flavocytochromes c$_3$ was carried out by hanging drop vapor diffusion in an air atmosphere at 4 °C in Linbro plates. Crystals were obtained with well solutions composed of 100 mM Tris-HCl buffer (pH 7.8 – 8.5, measured at 25 °C), 80 mM NaCl, 16 – 19% polyethylene glycol 8000, and 10 mM fumarate. Hanging drops (4 μl volume) were prepared by adding 2 μl of 7 mg/ml protein (in 10 mM Tris-HCl, pH 8.5) to 2 μl of well solution. After ~10 days, needles of up to 1 × 0.2 × 0.2 mm were formed. Crystals were immersed in a solution of 100 mM sodium acetate buffer, pH 6.5, 20% polyethylene glycol 8000, 10 mM fumarate, and 80 mM NaCl containing 23% glycerol as a cryoprotectant before mounting in nylon loops and flash-cooling in liquid nitrogen. For the E378D mutant enzyme a data set was collected to 1.7 Å resolution at DESY in Hamburg (Bruker BW7B; λ = 0.87 Å) using a MAR image plate detector. Crystals were found to belong to space group P2$_1$ with cell dimensions $a = 45.961$ Å, $b = 92.879$ Å, $c = 79.486$ Å, and $β = 91.17°$. For the R381K mutant enzyme a data set was collected to 2.1 Å resolution at SRS Daresbury (space group 142; $λ = 1.488$ Å) using an ADSC Quantum 4 detector. Crystals were found to belong to space group P2$_1$, with cell dimensions $a = 45.228$ Å, $b = 91.362$ Å, $c = 78.193$ Å, and $β = 91.26°$. Data processing was carried out using the HKL package (32). The wild-type flavocytochrome c$_3$ structure (1QJD) stripped of water was used as the initial model for molecular replacement. Electron density fitting was carried out using the program TURBO-FRODO (33). Structure refinement was carried using Refmac5 (34). The atomic coordinates have been deposited in the Protein Data Bank, entries 2B7R (E378D) and 2B7S (R381K).

**RESULTS**

**Characterization of Mutant Enzymes**—The molecular masses of mutant enzymes were determined by electrospay mass spectrometry (error of ≤ ± 5 Da). In comparison to wild-type (63,033 Da), the mass difference was found to be −16 Da for the E378D enzyme (expected difference, −14 Da), −29 Da for the R381K enzyme (expected difference, −28 Da), and −21 Da for the R381M enzyme (expected difference, −25). Each mutation was further verified by DNA sequencing. The average FAD content for the mutant enzymes was found to be 65% (E378D), 78% (R381K), and 60% (R381M). This compares with the value for the recombinant wild-type enzyme of ~73%. All catalytic rates were corrected for the variation in FAD content.

The abilities of the different variants to catalyze fumarate reduction were determined over a range of pH values. The resultant $k_{cat}$ and $K_m$ parameters are listed in Table 1, where it can be seen that all substitutions have greatly decreased the activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type. Substitution of the glutamate side chain by aspartate has lowered activity of the mutant enzymes relative to the wild type.
E378D-Fcc₃ enzyme shows there to be an essentially uniform decrease in efficiency of 10⁴-fold across the pH range measured. Proton inventory curves at pH 7.2 (where L = H or D) are shown in Fig. 2 and indicate that data for the wild-type and mutant enzymes fit to the equation

$$k_n = k_0 \left(\frac{k_D}{k_H}\right)^n$$

where $k_n$ is the rate constant in mixed isotopic solvent, which is given by $k_0$ (the rate constant for pure H₂O) corrected for the number of contributing hydrogenic sites, $n$. Thus, the data are described by the multiple exchangeable hydrogenic sites model of Schowen and Schowen (29) in which there are many hydrogenic sites in both the reactant and transition states. Such a model is entirely consistent with a proton pathway involving multiple exchangeable groups. The overall SKIE for wild-type and mutant enzymes were determined at pH values of 6.0, 7.2, and 9.0 (Table 2). It is clear from Table 2 that the SKIE is greatest at pH 6.0 and decreases with increasing pH. This is expected because there is a higher degree of protonation/deuteration at low pH. From the size of the SKIE it is evident that proton delivery is rate-limiting in the wild-type and mutant enzymes. Substitution of the glutamate side chain of Glu-378 by aspartate results in a doubling of the solvent kinetic isotope effect, whereas the substitution of the surface arginine with a lysine or a methionine has little effect on the overall SKIE.

### Table 1

| pH  | Wild-type | E378D | R381K | R381M | Wild-type | E378D | R381K | R381M |
|-----|-----------|-------|-------|-------|-----------|-------|-------|-------|
| 6.0 | 658 ± 34  | 1.27 ± 0.05 | 16.0 ± 0.4 | 0.49 ± 0.05 | 43 ± 10 | 72 ± 13 | 99 ± 7 | 6.6 ± 0.9 |
| 7.2 | 509 ± 15  | 2.05 ± 0.11 | 8.0 ± 0.5 | 2.20 ± 0.20 | 25 ± 2 | 319 ± 63 | 35 ± 5 | 8.7 ± 2.8 |
| 7.5 | 370 ± 10  | 2.43 ± 0.07 | 5.0 ± 0.4 | 2.80 ± 0.20 | 28 ± 3 | 549 ± 54 | 33 ± 7 | 4.7 ± 1.1 |
| 9.0 | 210 ± 13  | 5.92 ± 0.14 | 1.2 ± 0.1 | 2.29 ± 0.11 | 7 ± 2 | 1490 ± 120 | 5 ± 1 | 13.9 ± 3.0 |

* Values for the wild-type enzyme taken from Rothery *et al.* (38).

### Figure 2

Proton inventory curves showing the effect on $k_{cat}$ of the increasing percentage of deuteration of the assay buffer for wild-type enzyme (A), E378D mutant enzyme (B), R381K mutant enzyme (C), and R381M mutant enzyme (D). All kinetic measurements were carried out at pH = 7.2, 25°C, $I = 0.5 M$ and under saturating fumarate conditions (1 mM). The curves represent fits of the data to a model corresponding to multiple hydrogenic sites in reactant and transition states, as described by Schowen and Schowen (29).
nique reveals thermodynamics and kinetics in the same experiment and allows exquisite control of electron transfer and coupled reactions in the time and potential domains, thus complementing conventional solution studies (35). Studies on R381M and E378D were restricted because films were less stable (R381M) or revealed an additional FAD signal (E378D), suggesting some slight loss of FAD. In the absence of fumarate, all voltammograms showed a complex signal consisting of an oxidation and reduction peak (Fig. 3A), each of which contains overlaid components due to the oxidation and reduction of the four hemes and the FAD (26). The important point is that the FAD undergoes a cooperative two-electron reaction and, thus, gives rise to a much more prominent signal than the four hemes; consequently, the average position of the peaks in the oxidation and reduction direction corresponds (±10 mV) to the FAD two-electron potential (26). The pH dependences of the two-electron potential for WT, R381K, and E378D variants are shown in Fig. 3B, where in each case an excellent fit is obtained with a single $pK_{ox}$ close to pH 7. Below pH 7, i.e. pH < $pK_{ox}$, the two electrons are accompanied by transfer of only one proton, which must reside on the FAD, whereas above pH 7 (pH > $pK_{ox}$) an additional proton is transferred. Because oxidized FAD does not bind a proton directly, we have argued that the $pK_{ox}$ corresponds to a nearby, as yet unidentified base (26). Importantly, the two-electron reduced FAD binds one proton throughout the entire pH range. Reduction potentials (mV ± 10) at pH 7 and $pK_{ox}$ values (±0.2) are, respectively, −155 and 7.0 for wild-type, −168 and 7.2 for R381K, −168 and 6.8 for R381M, and −170 and not determined for E378D.

Fig. 4 shows for wild-type and R381K how the FAD peak positions recorded in the directions of increasing and decreasing electrode potential vary with scan rate. These diagrams are known as “trumpet plots,” and they reveal the kinetics of electron transfer and the way that electron transfer is coupled to other processes (35, 36). These experiments were carried out at a lower temperature, 5 °C, to observe signals in the presence of fumarate (see below), and the reduction potentials are slightly more positive. The symmetrical shapes in each case show that even at 100 V s$^{-1}$ (corresponding to the ms timescale) two electrons and one proton are transferred rapidly to and from the FAD with no complication from relatively slow processes. At pH 7, the exchange rate constants $k_{ex}$ (corresponding to the rate at which the two electrons and one (or two) protons transfer back and forth at zero over-potential) are 300 and 290 s$^{-1}$ for WT and R381K, respectively. Similar results were obtained irrespective of whether the cycle was initiated from the high potential or low potential limit. Also shown in Fig. 4 are the results obtained in the presence of 600 mM fumarate, sufficient to saturate the active site in all cases. At low scan rates, the peak-type signals are not observed because electrocatalytic fumarate reduction converts the signals to sigmoidal-like catalytic waves, for which the current is directly proportional to the turnover rate (indeed, the large solvent isotope effect measured in the solution steady-state studies was also manifested in the magnitude of the catalytic current, and SKIE values (pL) of 10.5 (6.3), 8.4 (7.3), and 6.7 (8.7) were measured for wild-type enzyme). The data for WT and R381K commence from the lowest scan rate, at which a clear FAD peak-type signal replaces the sigmoidal wave, i.e. when the cycle rate is sufficiently fast to outrun turnover (37). As expected, peaks emerge for the R381K mutant at a low scan rate (just above 10 V s$^{-1}$) whereas for the WT enzyme the peaks only become apparent above 70 V s$^{-1}$. In both cases the data points obtained in the presence of fumarate lie within those measured without substrate, and the reduction potential is barely changed. This is similar to the results obtained for E. coli fumarate reductase subunits A and B, although as with that enzyme, a negative shift was observed when succinate was present instead of fumarate.

Crystal Structures of the Mutant Flavocytochromes $c_{f}$—Attempts to obtain diffracting crystals of the R381M mutant enzyme were unsuccessful. However, good crystals were obtained for the E378D and R381K flavocytochromes $c_{f}$.
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Data sets to 1.7 Å (E378D) and 2.1 Å (R381K) resolution were used to refine the structures to final R-factors of 15.65% (E378D: R_free = 18.03%) and 14.41% (R381K: R_free = 19.67%) (Table 3). For each mutant enzyme the final model consists of one protein molecule, comprising residues 1–568, four hemes, the FAD, one fumarate molecule, and one sodium ion. In addition there are 1084 water molecules in the E378D model and 773 waters in the R381K model. Three residues at the C terminus of the protein (residues 569–571) could not be located in the electron density map for either structure. The root mean square deviation fit for all backbone atoms of the wild-type and E378D enzymes is 0.5 Å and for the wild-type and R381K enzymes it is 0.3 Å, indicating no major differences between the structures. The bound fumarate in the structures of the E378D and R381K enzymes is found in the same twisted conformation as observed for the hydroxylated malate-like species found at the active site of the wild-type enzyme. In the structures of the two mutant forms of the enzyme, the major changes observed are those resulting from the mutagenesis. Fig. 5 shows the electron density surrounding the proton pathway in the E378D and R381K enzymes, whereas Fig. 6 shows an overlay of this region in both mutant enzymes and the wild-type flavocytochrome c3. It can be seen that in the R381K enzyme the Lys-381 side chain is some 6.0 Å from Glu-378 but that two water molecules (Wat-508) have been introduced between the two residues. In addition, the side-chain carboxylate of Glu-378 is rotated around 90° relative to the position occupied in the wild-type enzyme.

In the E378D enzyme the position of Asp-378 is such that one of the side-chain carboxylate oxygens is in the same position as that taken by one of the glutamate oxygens in the wild-type enzyme. The position occupied by the second side-chain oxygen of Glu-378 in the wild-type enzyme is taken by a water molecule (Wat-175) in the structure of the E378D enzyme. This is shown in Fig. 6.

DISCUSSION

The movement of protons through a protein scaffold is often essential for enzyme-catalyzed reactions. Significant rates of proton transfer to a buried active site in an enzyme may require precisely oriented side chains and, in some cases, bound water molecules. The active site for fumarate reduction in flavocytochrome c3 is buried some 10 Å from the surface of the protein. Conversion of fumarate to succinate requires the equivalent of two electrons and two protons; these are supplied in the form of a hydride, donated from the flavin N5 to the fumarate C2, and a proton, donated from the active-site acid, Arg-402, to the fumarate C3 (13, 16–18). The mechanism for fumarate reduction by Fcc3 is shown in Fig. 7. Previous mutagenesis studies have conclusively shown Arg-402 to be the active-site acid, and there-
fore, the translocation of protons from the solvent to this active-site acid is required for subsequent catalytic cycles (16, 17, 27). It has been proposed that the delivery of protons to the substrate occurs via a pathway involving three residues, Arg-381, Glu-378, and Arg-402 (15–18, 26), and this pathway is shown in Fig. 1. It is interesting to note that these three residues are strictly conserved throughout the fumarate reductases and succinate dehydrogenases, consistent with a common mechanism operating in all members of this enzyme family. To investigate the importance of this pathway, we have used a mutagenesis approach to perturb it.

Altering the glutamate side chain by substitution with the shorter chain-length aspartate residue (E378D) shows some interesting results. It is immediately clear from the crystal structure of E378D that the proposed proton transfer pathway remains essentially intact and that there have been only small movements in the positions of crucial residues. Importantly, within error, there are no changes in the distances between the flavin N5 and the fumarate C2 or between the fumarate C3 and the side chain of Arg-402. From Fig. 6, it is apparent that the Asp-378 carboxylate group does not exactly overlay that of Glu-378. In fact only one of the side-chain carboxylate oxygens of Asp-378 is in the same position as that taken by one of the Glu-378 oxygens in the wild-type enzyme. Even so, proton transfer distances from Asp-378 remain similar to those seen for Glu-378 in the wild-type enzyme; that is, 3.1 Å to Arg-402 (3.1 Å in wild-type) and 3.5 Å to Arg-381 (3.1 Å in wild type). Although these changes are not huge, it is likely since the proton transfer rate is exponentially related to distance that even small alterations in proton transfer distances may lead to large changes in proton transfer rates.

The crystal structure of the R381K mutant enzyme also shows some intriguing features. It is evident that substitution of the branched guanidine group of Arg-381 by the amine group of Lys-381, so close to the surface of the protein, has enabled two water molecules to be incorporated within the proposed proton transfer pathway (Fig. 5b). The proton transfer distance from Arg-402 to fumarate C3 is unchanged in this mutation at 3.0 Å. However, the Glu-378 side chain has rotated by ~90° and is now 3.0 Å from Arg-402. Although the distance between Glu-378 and Lys-381 is now large, 6.0 Å, the 2 water molecules incorporated into the pathway are likely to mediate proton transfer between Lys-381 and Glu-378. Indeed it is possible that protons may be picked up by Glu-378 directly from water, bypassing the need for Lys-381 altogether. Support for this idea comes from the fact that the R381M mutant enzyme, in which the methionine side chain would be unable to participate in proton transfer, has a $k_{cat}$ value (at pH 7.2) only 4-fold less than that for the R381K enzyme (Table 1) and in addition has a similar SKIE value (Table 2). Unfortunately we were unable to obtain crystals of the R381M mutant enzyme, and so structural comparisons between the two mutants cannot be made.

Steady-state kinetic analyses of the mutant enzymes reveal that, whereas they all retain measurable activities, there are orders-of-magnitude decreases in $k_{cat}$. This is perhaps not surprising given the importance of these residues as key components of the proton transfer pathway. This is made even more apparent if we consider the solvent isotope effects. It was previously reported that the wild-type enzyme exhibited a large

![FIGURE 5. Stereoviews of the electron density surrounding the proton delivery residues in the E378D (a) and R381K (b) mutant flavocytochromes c3. The orientation is identical to that used in Fig. 1. The electron density maps were calculated using Fourier coefficients $2F_o - F_c$ where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively, the latter based on the final model. The contour level is 1σ, where σ is the root mean square electron density. This figure was generated using BOBSCRIPT (39) and RASTER 3D (40).]
SKIE (27), with a value of around 8 at pH 7.2. This was taken as clear evidence that proton transfer, not electron transfer, was rate-limiting in Fcc3. Thus, one would expect that any disruption in proton delivery would have a serious effect on the rate of the enzyme-catalyzed reaction. In the case of the R381K and R381M mutant enzymes there are 60- and 230-fold falls, respectively, in the values of \( k_{cat} \) at pH 7.2. Also, the SKIEs (at all values of \( p_L \)) are the essentially the same, within error, as that seen for the wild-type enzyme, i.e. proton transfer remains rate-limiting in both these mutant enzymes. The effects are no less dramatic for the E378D enzyme, where at pH 7.2 there is a 250-fold decrease in \( k_{cat} \) and a doubling of the SKIE value. Thus, the E378D mutation would appear to have made proton transfer even more rate-limiting, suggesting it plays the central role in proton delivery to the active-site acid. This is consistent with the structural changes in the proton pathway seen from the crystal structure.

The protein film voltammetry experiments provide a different perspective on the coupled proton transfers. The fast scan experiments reveal details of the electron and proton transfers that occur before the catalytic cycle can be completed (37); in this case we see that the rates at which two (both) electrons and either one or two protons (below and above \( pK_{ox} \), respectively) do not differ significantly between WT and R381K enzymes. This result shows clearly that the first proton in the catalytic cycle is transferred to the FAD by a pathway that does not involve R381K. This is the proton that is then transferred to fumarate as a hydride and must be partly responsible for the large isotope effect. The proton transfer pathway that depends upon Arg-381 comes into operation only when the second proton is required to complete the catalytic cycle.

It is interesting to note that a glutamate residue (Glu-25) has also been proposed to play a central role in the proposed proton transfer pathway in a [NiFe] hydrogenase (41). Indeed, in this case it has been proposed that the glutamate residue acts as an essential proton transfer “gate” during the catalytic cycle of the enzyme. Not surprisingly, the Glu-25→Asp mutation in [NiFe] hydrogenase also causes large effects on proton transfer, and the authors suggest the possibility that this may be due to the lower mobility of the aspartate lateral chain compared with glutamate (41). The implication of this explanation is that the motion of the glutamate is the critical factor for proton transfer in [NiFe] hydrogenase, and it is possible that this may also be the case in flavocytochrome \( c_3 \).

In addition to the SKIE values, it is also worth considering the proton inventories. At its best a proton inventory can allow the determination of the number of protons undergoing changes in a rate-determining step and sometimes can even allow the breakdown of the overall SKIE into its reactant state and transition state components (29). In the simplest case this can give rise to a linear relationship between the observed rate constant and the mole fraction of \( D_2O \), indicating that only one proton is involved in the transition state; more often though, a curve is observed indicating the involvement of more than one proton. At its most complicated, a curve is seen that corresponds to multiple exchangeable hydrogenic sites. In
such a case it is not possible to give the exact number of protons involved, but it does indicate that many exchangeable sites exist. This is the case seen for Fcc, and the E378D and R381K mutant enzymes (Fig. 2). Although this is not proof of a proton translocation pathway, it is certainly consistent with it. Thus, we believe that the large SKIE values for both wild-type and mutant enzymes alongside the complex proton inventories give good support to the idea of the proposed proton pathway.

In conclusion we have demonstrated that during the catalytic cycle of fumarate reductase, residues Arg-381 and Glu-378 in conjunction with the active site acid Arg-402 are essential for the efficient delivery of one proton to the catalytic intermediate formed at the active site and together form a proton pathway, fully conserved throughout the fumarate reductase/succinate dehydrogenase family. The other proton required in the catalytic cycle is transferred to the FAD via another mechanism, most likely directly from solvent, and does not use the Arg-381/Glu-378/Arg-402 pathway.

Acknowledgments—We thank SRS Daresbury for the use of synchrotron facilities. Synchrotron access at EMBL Hamburg was supported by the European Community Access to Research Infrastructure Action of the Improving Human Potential Programme to the EMBL Hamburg Outstation, contract number HPRICT-1999-00017.

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