must be very low at temperatures near 0°C.
14. P. Zwick, *Handb. Zool. (Berl.)* **4**, 1 (1980).
15. A. K. Brodskiy, *Entomol. Obozr.* **61**, 491 (1982).
16. C. H. Nelson, *Ann. Entomol. Soc. Am.* **77**, 466 (1984).
17. Video segments in platform-independent QuickTime format can be obtained through the World Wide Web (http://cac.psu.edu/~jhm10); or anonymous FTP server (FTP.CAC.PSU.EDU/pub/people/marden); or Gopher (Penn St. Univ./Info. servers at Penn St./Research Centers and Insts./Inst. Mol. Evol. Genetics/Faculty/Marden).
18. A. R. Gaufin, W. E. Ricker, M. Miner, P. Milam, R. A. Davis, *Trans. Am. Entomol. Soc.* **98**, 1 (1972).
19. J. Kukalova-Peck, "Origin and evolution of insect wings and their relation to metamorphosis as documented by the fossil record," figure 35, in *J. Morphol.* **156**, 53–126 (1978).
20. Statistics for regression lines are as follows, where y refers to the ratio of surface-skimming velocity to swimming velocity, and x refers to the ratio of wing length to body length: 1° to 2°C, $y = 6.26x - 2.27$, $r^2 = 0.81$, $P < 0.0000$; 6° to 8°C, $y = 13.52x - 4.19$, $r^2 = 0.94$, $P < 0.0000$; 16° to 18°C, $y = 13.20x - 1.20$, $r^2 = 0.72$, $P = 0.0038$; and 20° to 23°C, $y = 17.40x - 3.05$, $r^2 = 0.74$, $P = 0.0006$.
21. We thank J. P. Slusark for guiding us to a field site at which winter-emerging stoneflies are particularly abundant, and for species identification. We also thank D. M. Henderson for generously allowing us to use her high-speed video equipment and K. Dennison for laboratory assistance. This research was supported by NSF grant IBN-9317969.

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The Structure of Flavocytochrome c Sulfide Dehydrogenase from a Purple Phototrophic Bacterium

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The structure of the heterodimeric flavocytochrome c sulfide dehydrogenase from *Chromatium vinosum* was determined at a resolution of 2.53 angstroms. It contains a glutathione reductase–like flavin-binding subunit and a diheme cytochrome subunit. The diheme cytochrome folds as two domains, each resembling mitochondrial cytochrome c, and has an unusual interpropionic acid linkage joining the two heme groups in the interior of the subunit. The active site of the flavoprotein subunit contains a catalytically important disulfide bridge located above the pyrimidine portion of the flavin ring. A tryptophan, threonine, or tyrosine side chain may provide a partial conduit for electron transfer to one of the heme groups located 10 angstroms from the flavin.

Flavocytochrome c sulfide dehydrogenase (FCSD) from *Chromatium vinosum* catalyzes the reversible conversion of sulfide to elemental sulfur in vitro (*1*). Because of the nature of putative targeting sequences in the partial DNA sequence (*2*), the enzyme is believed to be located in the periplasm. It is a heterodimer with a relative molecular mass (M_r) of 67 kD, consisting of a flavoprotein subunit of M_r = 46 kD and a diheme cytochrome of M_r = 21 kD. The flavin-adenine dinucleotide (FAD) is bound covalently to the flavoprotein subunit by an 8-α-methyl(*S*-cysteinyl) thioether linkage (*3*). The diheme cytochrome subunit contains 174 residues which, based on heme-binding fingerprints in the amino acid sequence, appear to form two similar domains (*2*, *4*). When aligned, the two tandem domain sequences are only 7% identical (*4*). The DNA sequence for both the cytochrome subunit and the first 95 residues of the flavoprotein subunit is known (*2*). Residues 5 to 45 of the latter show significant similarity (15 to 39%) for the class of FAD-containing enzymes represented by glutathione reductase (GR) and lipoamide dehydrogenase. The site of covalent attachment of FAD (Cys^{42}) is close to the redox-active disulfide site in GR and to the sites of covalent attachment of FAD in succinate dehydrogenase and fumarate reductase (*4*).

Crystals of FCSD from *C. vinosum* were obtained as reported previously (*5*). Native data (95% complete and fourfold redundant to a resolution of 2.53 Å) were collected on a Hamlin multiwire area detector from two crystals and combined to yield an R_{merge} of 5.8%. The structure was solved by the multiple isomorphous replacement (MIR) method (*6*), and the partial deduced amino acid sequence (*2*) was fitted to the electron density map. The remaining 306 residues of the flavoprotein subunit, determined by classical sequencing methods (*7*), were then placed in the density and the structure was refined. The refined model (*8*) consists of two heterodimers with 9076 nonhydrogen polypeptide atoms, two FAD molecules, and four heme molecules, but no solvent. The crystallographic R factor ($\Sigma||F_o| - |F_c||/\Sigma|F_o|$) is 23.7% for all data between 8.0 and 2.53 Å. The root mean-square (rms) deviations from ideal bond lengths and angles are 0.017 Å and 3.8°, respectively.

The two heterodimers in the asymmetric unit are virtually identical. Each molecule (Fig. 1) consists of a cytochrome subunit containing two domains and a flavoprotein subunit containing three domains. The two subunits are tightly associated. The two domains of the cytochrome subunit are approximately equal in size and are similar in structure despite low sequence identity (Fig. 2).

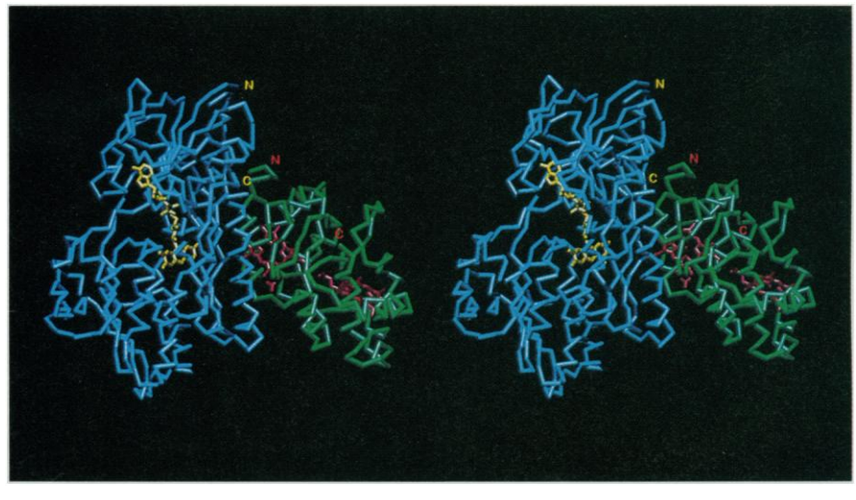

Fig. 1. Stereoview of a C_α tracing of the FCSD molecule with the flavoprotein subunit blue, the cytochrome subunit green, the flavin yellow, and the hemes red. The NH_2- and COOH-termini are labeled in yellow for the flavoprotein and in red for the cytochrome (*21*).

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They are related by an approximate twofold axis roughly perpendicular to the heme planes and passing near the propionic acid groups. Each domain consists of four α helices and intervening polypeptide loops that are wrapped around the heme group. The first and second domains contain 77 and 85 residues, respectively, and are connected by 12 residues. When the two domains are superimposed, the rms deviation between equivalent C_α positions is 2.20 Å, with eight residues inserted at four locations in the second domain. The edges of the porphyrin rings are 11.4 Å apart and the two iron atoms are separated by 19.0 Å. The heme planes are inclined to each other by about 30° and are oriented in a unique manner, with the propionic acid groups on ring A hydrogen-bonded to each other in the protein interior (Fig. 3). The other two propionates, on ring D, lie near the protein surface but are only partially exposed to solvent (9).

The first two domains of the flavoprotein subunit each contain a five-stranded parallel β sheet flanked on one side by three α helices and on the other side by a three-stranded antiparallel β sheet. The third domain consists of a three-stranded antiparallel β sheet followed by an α-helix (*10*). The flavin-bearing active site of FCSD is shown in Fig. 4. As predicted chemically (*3*), the site of covalent attachment of FAD is Cys^{42}. The *si* face of the flavin ring lies against the polypeptide backbone of residues 42 to 44. Above the pyrimidine portion of the flavin ring, opposite the *re* face, there is a disulfide bridge connecting Cys^{161} and Cys^{337} (*11*). The presence of the disulfide in FCSD was predicted on the basis of the kinetics of sulfite binding to the enzyme as a function of pH (*12*). At the N1-O2 locus of the flavin ring there is an α helix (Fig. 4) oriented with its NH_2-terminus pointing toward these atoms. The peptide nitrogens of Gly^{305} and Tyr^{306}, the second and third residues of the helix, form hydrogen bonds to N1 and O2, respectively. Surprisingly, there is no basic residue near this site, a situation that is unlike that seen in most other flavoenzymes (*13*) which, like FCSD (*1*), form an anionic flavin semiquinone, and is contrary to predictions based on redox potential measure-

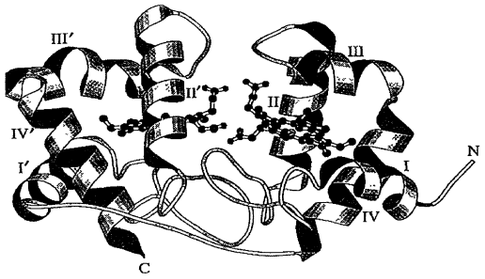

Fig. 2. Ribbon diagram of the diheme cytochrome subunit. The pseudo twofold axis relating the two domains is oriented vertically. Each domain contains four α helices labeled I to IV and I′ to IV′. This diagram was made using the program MOLSCRIPT (*22*).

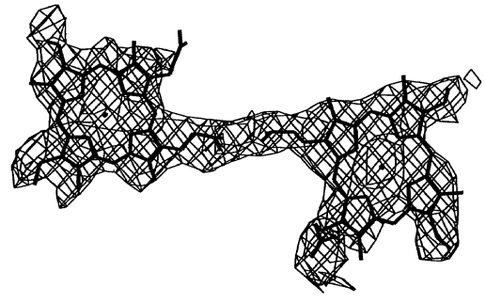

Fig. 3. Electron density surrounding the heme groups of the cytochrome subunit of FCSD. The map was obtained from MIR phases, computed at 3.0 Å resolution, and averaged about the noncrystallographic twofold axis after solvent flattening and histogram matching. The two propionic acid groups in the center of the molecule are hydrogen-bonded to each other.

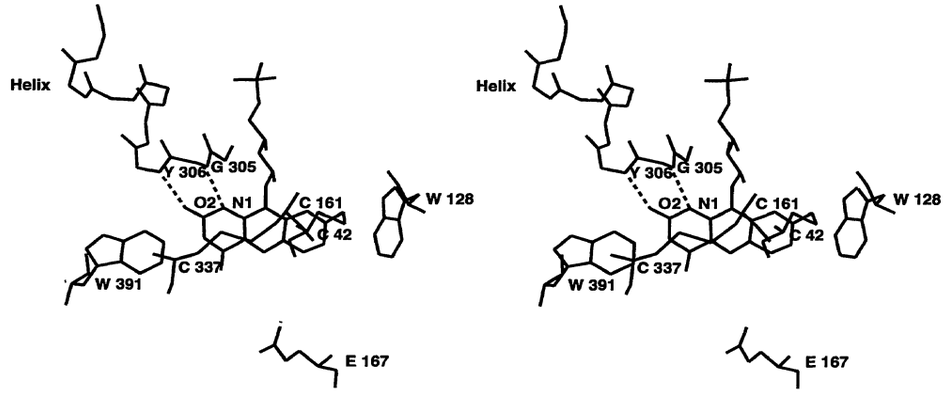

Fig. 4. Stereoview of the active site of the flavoprotein subunit of FCSD. The *re* face of the isoalloxazine ring and the ribityl group are shown, as well as key residues that interact with, or are close to, the flavin ring. The Cys^{161} and Cys^{337} disulfide bridge lies above the pyrimidine ring of the flavin, whereas Cys^{42}, the covalent attachment point of the flavin, lies below the benzenoid ring. The backbone of a nearby helix, which forms the only hydrogen bonds to the flavin ring, is also shown (*21*).

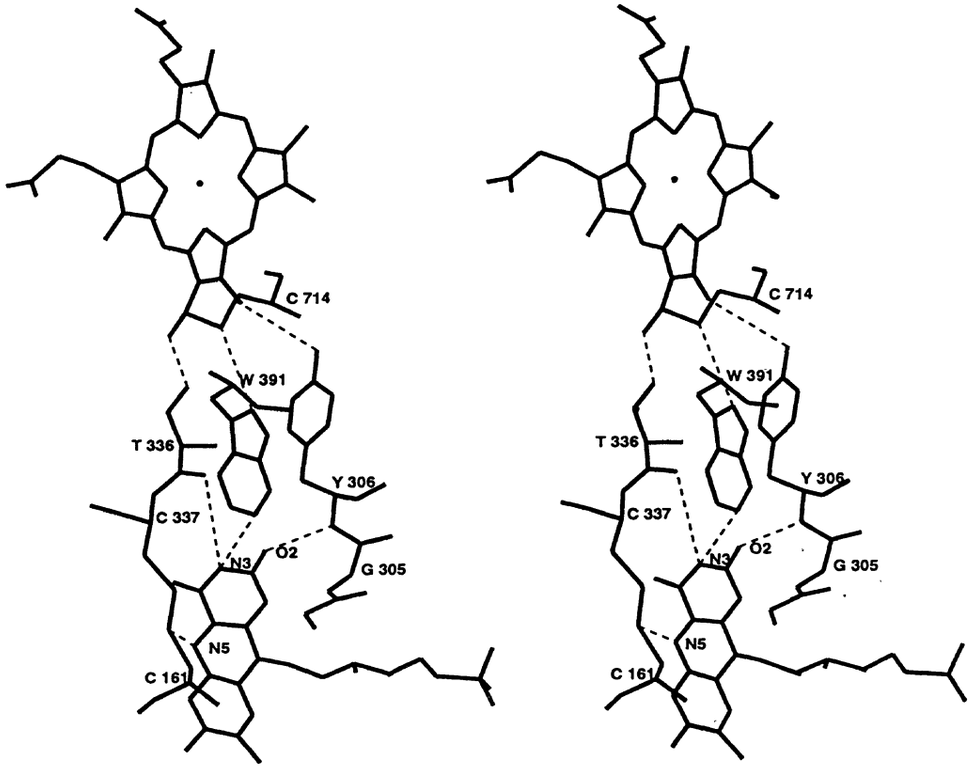

Fig. 5. Stereoview of four potential pathways for electron flow from the flavin to the heme of FCSD. Dashed lines indicate jumps through space or through a hydrogen bond between atoms of the polypeptide chain and the flavin or heme groups (*21*).

ments (*14*) and sulfite binding (*12*). Instead, the positive end of the dipole of the helix may be responsible for these properties of the flavin. There are two tryptophan residues near the flavin ring. Trp^{128} is close to the benzenoid ring, inclined to it by about 45°, and within 4.1 Å of C8M. Trp^{391} is located near the pyrimidine ring of the flavin, 3.2 Å from N3, and the tryptophan and flavin rings are roughly parallel. This tryptophan is likely to be responsible for charge transfer absorbance of the sulfite adduct (*12*), although the cystine disulfide could also fulfill this role. There are no side chain interactions with the flavin ring, other than the covalent bond connecting the 8-α methyl group to Cys^{42}. However, Glu^{167} is located 4.6 Å from N5 and might serve as an active site base for catalysis (*15*).

In the intramolecular electron transfer complex of FCSD, the pyrimidine portion of the flavin ring lies closest to the heme of the NH_2-terminal cytochrome domain. The planes of the heme and the flavin rings are inclined by about 20° to each other. The closest distance between the two prosthetic groups is 9.9 Å, from O2 of the flavin ring to the vinyl methylene atom (CBC) of pyrrole ring C of heme I. There are three side chains on the flavoprotein subunit, Trp^{391}, Tyr^{306}, and Thr^{336}, which are close to the heme group and may provide potential pathways for electron flow from flavin to heme (Fig. 5) (*16*). The indole ring of Trp^{391} lies between the flavin and the heme and is of interest because of its putative charge transfer function. The peptide nitrogen of Tyr^{306} is hydrogen-bonded directly to O2 of the flavin, providing a convenient path to the heme. Thr^{336} is about 4 Å from the flavin ring, providing a short link to the heme. It is also covalently connected to Cys^{337} and to Cys^{161}. If the Cys^{161}-Cys^{337} disulfide plays a redox-active role in catalysis, as suggested by the sulfite binding study (*12*), then this last path might be important. The interface between the flavoprotein and cytochrome subunits includes a surface area of about 1750 $Å^2$. The two surfaces are complementary, with a convex cytochrome surface contributed by helices I and III of domain I and a concave flavoprotein surface composed of the three β strands and a COOH-terminal helix in domain III (Fig. 1). There are 13 hydrogen bonds, including one salt bridge, connecting the two subunits.

The structure of FCSD differs fundamentally from the two other known flavocytochrome structures, flavocytochrome b_2 (FCB2) (*17*) and *p*-cresol methylhydroxylase (PCMH) (*18*). The structures of the three flavocytochromes (FCSD, FCB2, and PCMH) show a variety of cofactor interactions in electron transfer complexes. The angles between the heme and flavin planes are observed at values near 0° (FCB2), 20° (FCSD), and 60° (PCMH). This suggests that the interplanar angle between redox cofactors can vary substantially in electron transfer complexes and is not constrained to a "magic angle" as previously suggested (*19*). The portion of the flavin ring through which electron transfer may occur also differs considerably in the three flavocytochrome systems, being the N5, the N1-O2, and the C8M in FCB2, FCSD, and PCMH, respectively. Only the intercofactor distance of 8 to 10 Å remains relatively constant in the three systems.

REFERENCES AND NOTES

1. M. A. Cusanovich, T. E. Meyer, R. G. Bartsch, in *Chemistry and Biochemistry of the Flavoenzymes*, F. Muller, Ed. (CRC Press, Boca Raton, FL, 1991), vol. 2, p. 377.
2. M. M. Dolata, J. J. Van Beeumen, R. P. Ambler, T. E. Meyer, M. A. Cusanovich, *J. Biol. Chem.* **268**, 14426 (1993).
3. W. C. Kenney, D. E. Edmondson, T. P. Singer, *Eur. J. Biochem.* **48**, 449 (1974).
4. J. J. Van Beeumen *et al.*, *J. Biol. Chem.* **266**, 12921 (1991).
5. F. R. Salemme, *J. Mol. Biol.* **159**, 551 (1982).
6. The crystals are monoclinic, space group *C*2, with cell parameters a = 168.6 Å, b = 84.6 Å, c = 106.4 Å, and β = 107.0° and contain two flavocytochrome molecules per asymmetric unit. Data from five heavy atom derivatives obtained by soaking were recorded ... ding sites were identified by difference Patterson and difference Fourier analysis. The heavy atoms were distributed over 24 independent sites, some of which were common to several derivatives. All but two sites formed pairs related by twofold noncrystallographic symmetry. The exceptions, common to two derivatives, were located on the local twofold axis. A native anomalous difference Fourier synthesis calculated with MIR phases showed four iron sites per asymmetric unit, which also obeyed the noncrystallographic symmetry. The phasing power of the derivatives was poor below 3.7 Å resolution. The phases were improved by solvent flattening, histogram matching, and phase extension to 3.0 Å resolution with SQUASH [K. Y. J. Zhang, *Acta Crystallogr.* **D49**, 213 (1993)]. The final map was averaged about the noncrystallographic twofold axis with the use of the heavy atom and iron sites to determine the transformation matrix. The cytochrome and flavoprotein subunits could be traced, with only minor ambiguity, in a minimap of the averaged electron density. The two heme groups (centered on the irons) and the FAD could be visualized and their sites of covalent attachment identified. Approximate α-carbon positions were then transferred to the graphics system and used as a guide for fitting the published sequence of the cytochrome subunit and the first 95 residues of the flavoprotein subunit.
7. The sequences of residues 96 to 160 and of five additional polypeptide segments, obtained by protein sequencing methods, were aligned to the electron density map and fitted. The amino acid sequence of the first 160 residues of the flavoprotein subunit of FCSD were determined from Edman degradation of several Lys-C endoproteinase peptides. The first 95 of these were in agreement with the partial DNA sequence (*2*). The remainder of the protein sequence (residues 161 to 401) was derived from five nonoverlapping fragments (*20*). These fragments were aligned and verified by comparison with the electron density map obtained experimentally. In this step, extensive use was made of the anomalous scattering difference map of the native protein to help identify sulfur atoms.
8. The model was refined with X-PLOR [A. T. Brünger, *X-PLOR Manual*, version 3.0 (Yale Univ. Press, New Haven, CT, 1992)], and the resolution was extended to 2.53 Å by two cycles of simulated annealing followed by positional and temperature factor refinement. There are four positions where the amino acid sequence and side chain electron density disagree. These ambiguities should be resolved when the gene sequence becomes available and the refinement is complete. None of these positions is located in the active site.
9. The close proximity of the heme groups is consistent with circular dichroism studies that indicate a strong interaction between the heme groups (*1*). Of the eight oxygen atoms on the four heme propionic acid groups, only two, OD1 of heme 1 and OD2 of heme 2, are appreciably exposed to solvent, each having an accessible surface area approximately 40% of that calculated for the free heme. The two deeply buried propionates on rings A of the hemes are hydrogen-bonded to Tyr^{128} O^{η} (heme 1) and Arg^{141} $N^{\eta 2}$ (heme 2), in addition to each other. The more exposed propionates, on rings D of the hemes, form hydrogen bonds with Thr^{52} N, Thr^{52} O^{γ}, and Met^{54} N (heme 1) and with Glu^{144} N (heme 2).
10. The first two domains of the flavoprotein subunit show pronounced structural similarity to the FAD- and NADP-binding domains of GR [G. E. Schulz, R. H. Schirmer, W. Sachenheimer, E. F. Pai, *Nature* **273**, 120 (1978)]. The third domain is considerably smaller than the comparable domain (the "interface domain") of GR, although it is located in approximately the same place, carries out an analogous function (as an interface), and even shares the first two antiparallel β-strands with the corresponding domain of GR.
11. The disulfide does not correspond to the redox-active disulfide of GR (residues 58 and 63, next to the *si* face of the flavin). However, it is analogous to the redox-active disulfide of thioredoxin reductase (TRR), which is also located above the *re* face of the flavin ring [G. Waksman, T. S. R. Krishna, C. H. Williams Jr., J. Kuriyan, *J. Mol. Biol.* **236**, 800 (1994)], but it does not correspond in sequence to that of TRR.
12. The flavin of FCSD can bind sulfite, cyanide, mercaptans, or thiosulfate to form flavin N5 adducts which in turn form charge-transfer complexes with the protein (*1*) [T. E. Meyer, R. G. Bartsch, M. A. Cusanovich, *Biochemistry* **30**, 8840 (1991)]. The kinetics of sulfite adduct formation and decay as a function of pH are indicative of a complex process, and the experiments suggest the presence of four functional groups near the flavin, interpreted as a lysine, a histidine, a cystine disulfide, and an aromatic residue. An apparent pK_a of 8.5 for decomposition of the flavin-sulfite adduct was ascribed to the release of a sulfhydryl on reaction of sulfite with the disulfide.
13. S. Ghisla and V. Massey, in *Chemistry and Biochemistry of the Flavoenzymes*, F. Muller, Ed. (CRC Press, Boca Raton, FL, 1991) vol. 2, p. 243.
14. T. E. Meyer, R. G. Bartsch, M. S. Caffrey, M. A. Cusanovich, *Arch. Biochem. Biophys.* **287**, 128 (1991).
15. When ionized, Glu^{167} may also destabilize the flavin-sulfite adduct, a function previously attributed to a histidine residue, based on the apparent pK_a of 6.5 for reaction with sulfite at N5 (*12*).
16. The intramolecular electron-transfer rate constant from flavin to heme may be as high as 10^6/s [M. A. Cusanovich, T. E. Meyer, G. Tollin, *Biochemistry* **24**, 1281 (1985)]. The computer program Pathways II [J. J. Regan, *Pathways II Software v2.01* (Jeffrey J. Regan, San Diego, CA, 1993)] was used as an aid to locate potential paths for electron flow.
17. Z.-x. Xia and F. S. Mathews, *J. Mol. Biol.* **212**, 837 (1990).
18. F. S. Mathews, Z.-w. Chen, H. D. Bellamy, W. S. McIntire, *Biochemistry* **31**, 238 (1990).
19. H. Pelletier and J. Kraut, *Science* **258**, 1748 (1992).
20. J. J. Van Beeuman *et al.*, unpublished results.
21. This figure was made with the program Turbo-Frodo [A. Roussel and C. Cambillau, Turbo-Frodo, in *Silicon Graphics Geometry Partners Directory* (Silicon Graphics, Summit, NJ, 1991), p. 86].
22. P. J. Kraulis, *J. Appl. Cryst.* **24**, 946 (1991).

23. We wish to thank J. Fitch for protein purifications and R. Durley for helpful comments. Supported by USPHS grants GM-20530 (to F.S.M.) and GM-21277 (to M.A.C.) and by grant 2001891 from the Fund for Joint Basic Research (to J.V.B.). Coordinates for FCSD have been deposited in the Brookhaven Data Bank.

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