tation. In this crystal structure, the preference for syn versus anti dominates over any preference for *intra*molecular versus *inter*molecular.

The central location of the proton in the hydrogen bond suggests nearly equivalent pK_a's. Syn-anti directionality and microenvironmental electrostatics control carboxylate basicity. In water, benzimidazole is a stronger base than carboxylate.[12-16] The pK_a of carboxyl increases in less aqueous solvents and exceeds that of imidazolium.[17,18] In anhydrous environments, the Asp-His dyad will favor the neutral form rather than the zwitterionic. This point is illustrated by the crystal structure of a carboxylic acid-imidazole clathrate, which favors the zwitterion when hydrated and the neutral form when anhydrous.[19] The zwitterionic dyad has an anti-oriented carboxylate, but the neutral form has a syn-oriented carboxyl.

These results inform the controversy about charge-relay catalysis in serine proteases. Structural studies on these enzymes[20-22] suggest a zwitterionic dyad with a syn-oriented carboxylate. Most believe that the proton does not transfer from imidazolium to carboxylate and the dyad remains zwitterionic. Warshel et al.[23] propose that an ionized Asp electrostatically stabilizes the transition state. The pK_a of His increases during the enzymatic reaction,[24] and a proximal carboxylate increases the basicity of imidazole.[25]

Proton-inventory studies suggest two-proton catalysis for hydrolysis of natural substrates.[26-28] Schowen[29] questions the use of equilibrium data[20-22] as evidence for a zwitterion in the transition state. Substrate binding may change the protein microenvironment to favor repositioning or transfer of the proton in the dyad.[28]

The crystal structure of a nearly centrally located proton in a carboxyl-imidazole hydrogen bond suggests that a similar structure can exist in a transition state. Repositioning of the proton in a broad single-potential well[30] would produce a curved proton inventory just as proton transfer would.[31] The dyad remains zwitterionic, but at the transition state the O···N distance shortens and the proton moves to center. Proton transfer is not required for catalysis; strong hydrogen bonding will suffice.[32] This catalytic hydrogen-bonding mechanism reconciles the kinetic, structural, and computational results presented to date.

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Supplementary Material Available: Tables of atomic coordinates for **1**·$^1/_2H_2O$, bond distances, and bond angles and ORTEP drawings of molecules A and B (8 pages). Ordering information is given on any current masthead page.

(12) The individual K_a's of **1** in 80% methanol-water are too close to separate by titration.[13] The first ionization is mostly imidazolium, and the second is mostly carboxyl. The pK_a of 2-methoxybenzoic acid[15] is 4.09 in water. The pK_a of 2-(phenoxymethyl)benzimidazole is 4.34 in 48% ethanol-water.[14] From the pK_a's of other benzimidazoles in 48% ethanol-water versus water, we estimate a pK_a of 4.68 for **1** in water.

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Copper Coordination Geometry in Azurin Undergoes Minimal Change on Reduction of Copper(II) to Copper(I)

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It has long been suggested[1] that the copper coordination environment in blue copper electron transfer proteins[2] is a compromise between preferred states for copper(I) and copper(II), and that this should lower the activation energy for electron transfer. Here we provide direct crystallographic evidence that the copper site in azurin, from *Alcaligenes denitrificans*, undergoes minimal structural change on reduction, consistent with the requirements for fast electron transfer. This conclusion is based on crystallographic refinement of the reduced, copper(I), azurin structure, at 1.9-Å resolution,[3] and comparison with the structure of the oxidized protein, at similar resolution.[4,5]

Azurin was isolated and purified from cultures of *A. denitrificans* (NCTC 8582) and crystallized in its oxidized, Cu(II), state as described previously.[6] These crystals were then reduced by soaking in standard mother liquor (0.1 M phosphate, 75% saturated with ammonium sulfate) containing 0.1 M ascorbic acid, the final pH of this solution being 5.5. Reduction appeared complete in 4-5 h, but soaking was extended until 7 h. This procedure resulted in small changes in the unit cell dimensions

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Table I. Bond Distances and Angles for the Copper Sites of Reduced, Copper(I), and Oxidized, Copper(II), Azurin from *A. denitrificans*

	reduced (this work)			oxidized (ref 5)		
	1	2	mean	1	2	mean
			Bond Disances, Å			
Cu–O(45)	3.25	3.19	3.22	3.16	3.09	3.13
Cu–Nδ_1(46)[a]	2.17	2.09	2.13	2.08	2.09	2.08
Cu–Sγ(112)[a]	2.22	2.31	2.26	2.12	2.17	2.15
Cu–Nδ_1(117)[a]	2.05	2.05	2.05	2.01	1.99	2.00
Cu–Sδ(121)	3.21	3.25	3.23	3.12	3.10	3.11
			Bond Angles, deg			
O(45)–Cu–Nδ_1(46)	68	68	68	72	76	74
O(45)–Cu–Sγ(112)	105	103	104	103	104	104
O(45)–Cu–Nδ_1(117)	82	85	83	78	81	80
O(45)–Cu–Sδ(121)	142	144	143	146	148	147
Nδ_1(46)–Cu–Sγ(112)[a]	132	131	132	135	135	135
Nδ_1(46)–Cu–Nδ_1(117)[a]	100	108	104	101	108	105
Nδ_1(46)–Cu–Sδ(121)	77	79	78	79	75	77
Sγ(112)–Cu–Nδ_1(117)[a]	126	119	123	122	116	119
Sγ(112)–Cu–Sδ(121)	109	109	109	109	105	107
Nδ_1(117)–Cu–Sδ(121)	90	94	92	94	98	96
	Distance of Cu from Plane through Nδ_1(46), Sγ(112), and Nδ_1(117), Å					
	0.12	0.16	0.14	0.16	0.09	0.12

[a] Bond distances and angles within the trigonal plane.

and a slight loss of high-angle diffraction data, compared with those of the oxidized protein.[7] Nevertheless, X-ray diffraction data to 1.9-Å resolution (2θ = 47.8° for Cu Kα radiation) could be collected on a CAD4 diffractometer, using partial step scan routines.[5,8]

Eight crystals, each freshly reduced before data collection, were required, with each able to be used for approximately 3 days before significant reoxidation occurred (as judged by the first detection of a visible blue color). Structure refinement was by restrained least-squares methods, with manual checking and rebuilding on an Evans and Sutherland PS 300 interactive graphics system.[9]

The final refined model[10] of 1956 protein atoms (for the two azurin molecules in the asymmetric unit), 258 water molecules, and two SO_4^{2-} ions gives a crystallographic R factor of 0.166 for the 19 281 reflections with $F > 1.0\sigma_F$ in the resolution range 10.0–1.9 Å. The upper limit for the average error in atomic positions, from the variation of R with resolution, is estimated as 0.20 Å, but for well-ordered parts of the structure, such as the copper site, the error should be substantially less. Our estimate of the standard deviations in the copper site geometry, based on the level of agreement between the two independent azurin molecules,[11] is ~0.05–0.10 Å for Cu–ligand bond distances and ~3.0–5.0° for ligand–Cu–ligand bond angles.

The basic copper coordination geometry seen in the oxidized azurin structure[4,5] consisted of three strongly bound ligands (the thiolate sulfur of Cys 112 and the imidazole nitrogens of His 46 and His 117) approximately coplanar with the copper, in a distorted trigonal arrangement. Bond distances were 2.00–2.15 Å. Longer axial approaches of ~3.1 Å were made by the thioether sulfur of Met 121 and the peptide carbonyl oxygen of Gly 45. In reduced azurin, hardly any change is seen (Figure 1). The only change is a slight expansion of the copper site, with all bond distances, including the axial approaches to Gly 45 and Met 121, increased by between 0.05 and 0.1 Å (Table I). Although these increases are only of the order of the estimated standard deviation, the pattern is consistent; *all* copper–ligand distances, in *both* molecules, are similarly increased. Small changes occur in bond angles, although no pattern is apparent, and the displacement of the copper atom from the N_2S plane is essentially unchanged from the oxidized protein (Table I).

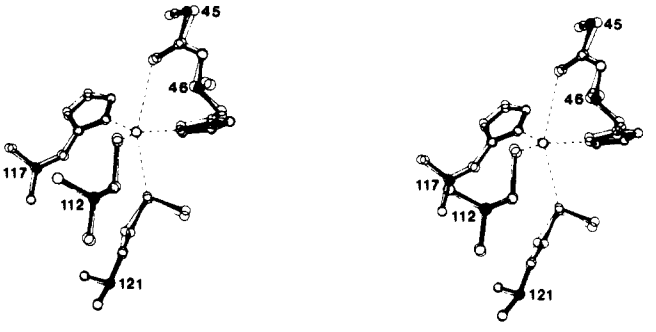

Figure 1. Stereoview of the copper site (molecule 2 in the asymmetric unit) showing the close correspondence between oxidized (full lines) and reduced (open lines) structures.

The Cu–N(His) and Cu–S(Cys) distances are similar to the values deduced from EXAFS studies of reduced azurin from *Pseudomonas aeruginosa*.[12] The Cu–S(Met) distance (3.23 Å) is, however, much greater than the EXAFS value of 2.70 Å. Because of this discrepancy, and because it seemed unlikely that the two azurins would differ so greatly, attempts were made to impose the EXAFS values on the copper site by tightly constraining the Cu–S(Met) distance during crystallographic refinement. No matter whether the copper, the Met side chain, or both were moved to conform to the EXAFS value, the structure always returned to that reported here and confirmed the Cu–S(Met) distance as ~3.2 Å.

The slight expansion of the copper site on reduction parallels the expected slight increase in the radius of copper(I), compared

(7) Cell dimensions: a = 75.1 Å, b = 73.8 Å, and c = 100.1 Å for reduced crystals, compared with a = 75.0 Å, b = 74.2 Å, and c = 99.6 Å for oxidized crystals. For the reduced crystals, only 23% of reflections in the outermost shell (1.9–2.1 Å) had $I > 2\sigma_I$, compared with 48% (in the shell 1.8–2.0 Å) for oxidized crystals.

(8) From an estimated 22 300 unique reflections to 1.9-Å resolution, 13 200 (i.e., 59%) were measured with $I > 2\sigma_I$ and 17 150 (77%) with $I > \sigma_I$.

(9) Refinement utilized the program PROLSQ (Hendrickson, W. A.; Konnert, J. A. In *Biomolecular Structure, Function, Conformation and Evolution*; Srinivasan, R., Ed.; Pergamon: Oxford, 1980; Vol. 1, pp 43–57), with restraints on bond lengths and angles (except those involving copper), planar groups, chiral volumes, torsion angles, and nonbonded contacts. Model building on the computer graphics system used the program FRODO (Jones, T. A. *J. Appl. Crystallogr.* **1978**, *11*, 268–272). In the final model the root-mean-square deviation from standard values was 0.015 Å for bond lengths and 3.2° for bond angles.

(10) Coordinates and thermal parameters have been deposited with the Brookhaven Protein Data Bank, Brookhaven National Laboratory, Upton, NY 11973. The copper site coordinates have also been deposited with the journal as supplementary material.

(11) Superposition of the two copper sites and their immediate surroundings (125 atoms) gives an rms difference in atomic positions of 0.19 Å. Comparison of copper bond lengths and angles between the two independent molecules suggests a lower error, however. Bond lengths differ by up to 0.09 Å (rms difference 0.06 Å) and bond angles by up to 8.0° (rms difference 3.9°).

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with copper(II). That this is the *only* change, with minimal change in bond angles, is consistent with the requirements for fast electron transfer, i.e., that the reorganization energy be low.[13] Similarly small geometric changes have been found between the redox-active reduced and oxidized structures of another blue copper electron transfer protein, plastocyanin,[14] and these data support the view that in both proteins the surrounding protein structure provides a copper site that is optimized for biological electron transfer. In azurin the copper ligands are tightly constrained by hydrogen bonding and van der Waals interactions, and the general region of the copper site appears the least flexible part of the molecule.[5] The principal copper ligands (one thiolate S^- and two imidazole N atoms) are a compromise between those favored by copper(I) and copper(II), and the geometry, trigonal with possible weak interactions with two axial groups, is intermediate between geometries favored by copper(I) (trigonal planar) and copper(II) (trigonal bipyramidal).

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Supplementary Material Available: Coordinates for the copper site of reduced azurin from *A. denitrificans* (2 pages). Ordering information is given on any current masthead page.

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Rearrangements and Stereochemistry of S_2 Additions to Olefins

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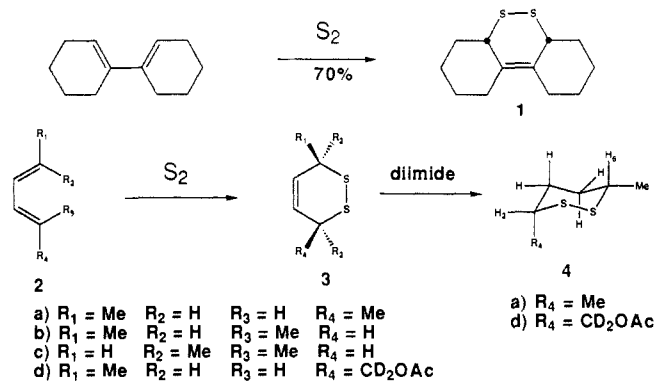
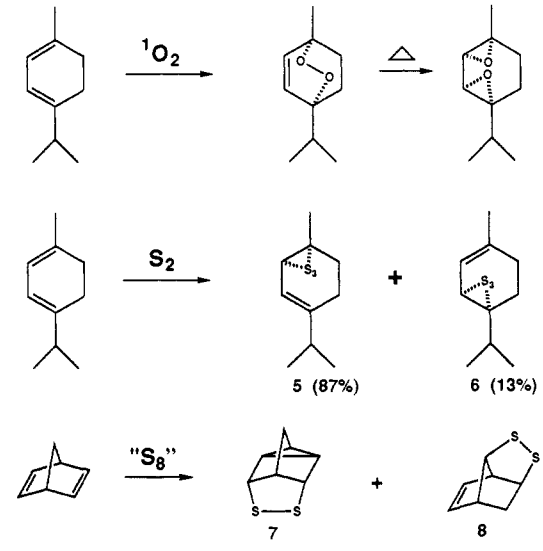
Since we first introduced S_2 chemistry in 1984,[1] several other laboratories have been stimulated to explore this new branch of organosulfur chemistry.[2] From our continuing work in this area, we can now report that the S_2 Diels-Alder type addition to acyclic 1,3-dienes occurs with stereochemical control that is consistent with the Woodward-Hoffmann rules.[3] Evidence is derived from the following expeirmental results. S_2 addition[1] to 1,1'-bicyclohexenyl (Scheme I) gives (70% yield) only the syn adduct **1** and to 2(*E*),4(*E*)-hexadiene (**2a**), the adduct **3a** (52% yield), also with 100% syn stereochemistry. The stereochemistry of **3a** was deduced from the 300-MHz ^1H NMR (CCl_2F_2) analysis of the product **4a** obtained (43% yield) from diimide[4] reduction of the double bond, using naphthosylhydrazine in refluxing diglyme. A single doublet (δ 1.34, J = 6.9 Hz) for the methyls is seen at 25 °C while at −25 °C (coalescence temperature of −10 °C, 12 kcal/mol barrier[5]) the presence of two doublets (δ 1.12 and 1.56) for the required axial, equatorial disposition of the methyls is confirmed.

Similarly, diimide reduction of the S_2-derived deuterated dithiin derivative **3d** yields (50%) only stereoisomer **4d**, in which the room-temperature 300-MHz ^1H NMR ($CDCl_3$) coupling constants (irradiation of the methyl signal at δ 1.4) for the two H_6 doublets (9.7 and 2.7 Hz) and for the two H_3 doublets (5.4 and 5.4 Hz) are consistent only with an equatorial methyl and axial acetoxy substituted deuterated methylene arrangement.[6] Although 2(*E*),4(*Z*)-hexadiene (**2b**) also affords (20%) only the syn adduct **3a** in an apparent violation of the Woodward-Hoffmann rules, a mechanism involving double-bond isomerization similar to the one proposed by O'Shea and Foote[7] (first proposed by Gollnick and Griesbeck[8]) for the analogous addition of $^1O_2$ to this diene is thought to be operative and is based on our observations from the S_2 additions to reactive olefins and cyclic 1,3-dienes described below.

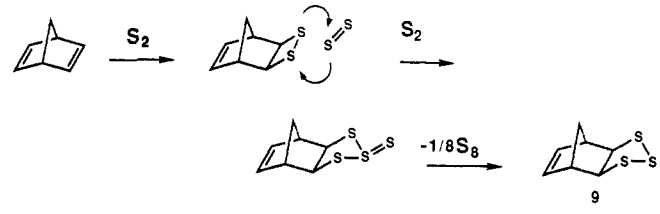
Scheme I



Scheme II



Scheme III



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