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Surface-Skimming Stoneflies: A Possible Intermediate Stage in Insect Flight Evolution

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Insect wings appear to have evolved from gills used by aquatic forms for ventilation and swimming, yet the nature of intermediate stages remains a mystery. Here a form of nonflying aerodynamic locomotion used by aquatic insects is described, called surface skimming, in which thrust is provided by wing flapping while continuous contact with the water removes the need for total aerodynamic weight support. Stoneflies surface skim with wing areas and muscle power output severely reduced, which indicates that surface skimming could have been an effective form of locomotion for ancestral aquatic insects with small protowings and low muscle power output.

Insects evolved the ability to fly approximately 330 to 400 million years ago, and they subsequently radiated and diversified to become the most speciose life form on the planet (*1*). How flight evolved in insects has been a topic of frequent debate (*2*), because the answer may yield valuable insight into reasons for insect diversification. Furthermore, flight exemplifies one of the great challenges for evolutionary biology, which is to determine transitions in function and selective advantage for intermediate stages during evolution of complex suites of interdependent anatomical, physiological, and behavioral features (*3*).

Fossils offer tantalizing clues regarding morphology and skeletal anatomy of certain primitive insect fliers (*4*); however, the fossil record is too sparse to resolve key phylogenetic or functional transitions. Recent debate has focused predominantly on the anatomical origin of wings (immovable thoracic lobes or articulated gills) and whether small protowings served originally for adaptive aerodynamic functions (*5*) or were used for thermoregulation and only subsequently became adapted for flight (*6*). Presently, the fossil, neurological, and developmental evidence (*4*, *7*) favors the wings-from-gills model (*2*); however, no previous hypotheses have offered a detailed model explaining how fliers could have evolved from swimmers, nor have they utilized detailed examinations of behavior, physiology, and morphology of the extant insect orders (Ephemeroptera and Plecoptera) that are anatomically and phylogenetically closest to preflight fossil insects.

Surface skimming, a wing-flapping mode of locomotion used by certain adult stoneflies (Plecoptera) and subadult mayflies (Ephemeroptera), is an attractive candidate for an intermediate stage between swimming and flying. Surface skimming consists of planar movement across a water surface, wherein propulsion is supplied by aerodynamic thrust, while continuous contact with the water removes the need for total aerodynamic weight support (Fig. 1). Thus, all components of the flight motor (wings, wing articulations, muscles, and neuromotor patterns) of primitive surface skimmers could have simultaneously undergone selection for incremental improvement in flapping aerodynamic performance. Here we present an experimental test of the hypothesis that incremental increases in wing size, flight musculature, and muscle power output bring about incremental improvement in the surface-skimming performance of a stonefly.

Stoneflies are weak-flying or nonflying aquatic insects that, except for wings, show relatively little morphological divergence from fossil ancestors dating back to the Carboniferous (Fig. 2) (*8*). *Taeniopteryx burksi* (Plecoptera: Taeniopterygidae) is a winter-emerging stonefly that is common across eastern and central North America, whose only conspicuous use of wing flapping in the field is for surface skimming (Fig. 1). In central Pennsylvania during February and March, *T. burksi* adults emerge and use surface skimming to cross open water whenever they emerge at a distance from shore (on emergent mid-stream rocks, sticks, or ice). After exiting the stream, adults feed terrestrially and arboreally on algae, and they mate (*9*). We have observed thousands of individuals in the field (ambient temperature 0° to 12°C) but have never seen one fly.

We videotaped surface-skimming locomotion of normal and wing-clipped individuals in the lab to determine how skimming velocity is affected by relative wing size, flight muscle ratio (the ratio of thoracic muscle mass to total body mass, a strong determinant of performance in flying insects) (*10*), temperature (which affects muscle power output in ectotherms) (*11*), and body size (*12*). Skimming velocity increased in a continuous, incremental fashion with increasing temperature, relative wing area, and flight muscle ratio (Table 1 and Fig. 3), reaching speeds as high as 44 cm/s. Surface skimming was effective even at temperatures as low as 1.5°C (Fig. 3), when muscle power output of ectothermic insects is severely restricted (*11*, *13*), and with wing size reduced to as little as 20 to 30% of normal (Figs. 1C and 4).

Unlike their behavior at cold ambient temperatures in the field, most *T. burksi* adults do attempt to fly in the lab (air temperature = 22°C). We classified performance of 31 flight-willing individuals as either (i) able to gain altitude in a sustainable fashion, (ii) able to sustain only level flight, or (iii) unable to sustain level flight while flapping. Only 6 individuals (19%) gained altitude, 9 (29%) sustained level flight, and 16 (52%) consistently lost altitude. These performance groups differed in mean flight muscle ratio but not in wing loading (Table 2).

In contrast to these results for morphological determinants of flight performance, our surface-skimming experiments showed that performance increased steadily with increasing wing area, up to the highest relative wing areas observed (Fig. 3). Similarly, flight

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muscle ratios in excess of 30% of body mass continued to improve surface-skimming performance (Fig. 3), whereas *T. burksi* can achieve level flight with flight muscle ratios averaging 25% (Table 2).

In summary, these results demonstrate that ancestral aquatic insects with small protowings, rudimentary thoracic muscles, and low muscle power output could have been effective surface skimmers, and that directional selection for faster surface skimming could have resulted in levels of elaboration of the flight motor that match or exceed requirements for powered flight.

An alternative interpretation is that surface skimming represents only an emergency response to occasional, accidental contact with water, made possible by the presence of motor patterns and anatomical features that evolved for aerial locomotion. However, the generally archaic morphology of stoneflies (Fig. 2), in combination with what appear to be specific and perhaps ancient adaptations for surface skimming, argues against this possibility. In *T. burksi*, the entire wing surface is covered with a dense mat of hairs that resist wetting (Fig. 5A). Hairs of strikingly similar structure are present in the subimago stage of mayflies (Ephemeroptera) (Fig. 5B) but are absent in the mayfly imago (Fig. 5C). Mayfly subimagos emerge on the water surface from aquatic nymphs and must escape the surface tension for their initial brief flight away from the water. An adult molt (unique among insects) then occurs, after which imagos have little or no contact with water and thus have little need for wet-resistant hairs. We hypothesize that the wing hairs of stoneflies and mayfly subimagos were inherited from a common surface-skimming ancestor, and that these hairs have been lost in more derived forms (including the mayfly imago stage and terrestrially emerging Odonates) as a result of selection for reduced aerodynamic drag or wing weight. The ventral tarsi (terminal leg segments) of *T. burksi* are also covered with a mat of specialized hairs that are the main point of contact with the water during surface skimming. Examination of Carboniferous fossil insects for hairs on the wings and tarsi might provide evidence that these forms used surface skimming before the evolution of flight.

Whether *T. burksi* represents a marginal flier whose flight capacities have evolved progressively from nonflying progenitors or digressively from more strongly flying ancestors is uncertain. Descriptions of stonefly flight capacities are so sparse that it is not presently possible to map flight ability on a phylogeny of stoneflies and thereby infer likely evolutionary transitions. To the extent that we can presently determine, Taeniopterygidae and related families (superfamily Nemouroidea) (*14*) are all either flightless or marginally capable fliers. Nemouroidea are thought to have undergone an evolutionary reduction in wing venation and flight ability (*15*); however, this conclusion is based on the presence of powerful flight musculature (which we have shown here to be a likely product of selection for surface-skimming performance) and the presupposition that all extant stoneflies evolved from fully flight-capable ancestors. Phylogenetic analyses place the su-

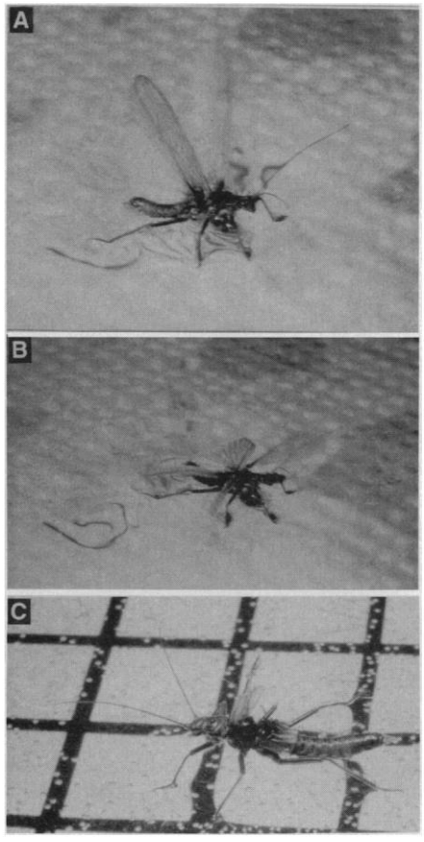

Fig. 1. Surface skimming in the laboratory by *T. burksi* stoneflies. (**A**) Shortly after initiation of the wing downstroke. (**B**) Near the start of the wing upstroke. All six legs, as well as the posterior abdomen in certain individuals, remain in continuous contact with the water surface at all points of the stroke cycle. For eight individuals filmed during surface skimming at room temperature (22°C) with high-speed video (500 images per second), wingbeat frequency averaged 37.3 Hz (SD = 3.3), and stroke amplitude was approximately 60° to 90° (wing tips contact the water at the bottom of the downstroke). (**C**) An individual with wing area experimentally reduced to 20% of normal wing area. A 1-cm^2 grid beneath the water shows approximate scale. Digitized segments of high-speed video are accessible through the Internet (*17*).

Table 1. ANOVA results showing determinants of stonefly surface-skimming velocity. For the whole model, $r^2 = 0.82$.

Source	df	Mean square	*F* ratio	*P*
Whole model	5	967	33.9	0.0000
Individual factors				
Temperature	1	875.0	30.7	0.0000
Body size (fresh mass)	1	1.1	0.04	0.84
Wing loading (N/cm^2)	1	408.2	14.3	0.005
Flight muscle ratio	1	241.0	8.5	0.006
Wing treatment (normal, clipped)	1	661.2	23.2	0.0000
Error	37	28.5		

Table 2. Differences in wing loading and flight muscle ratio (FMR) among flight performance categories. Wing loading is expressed in units of millinewtons per square centimeter.

Flight classification	Number	Mean wing load (SD)	Mean FMR (SD)
Unable to sustain level flight	16	0.095 (0.014)	0.221 (0.06)
Able to sustain level flight	9	0.099 (0.011)	0.251 (0.05)
Able to steadily gain altitude	6	0.087 (0.004)	0.300 (0.02)
		$P = 0.18$	$P = 0.01$

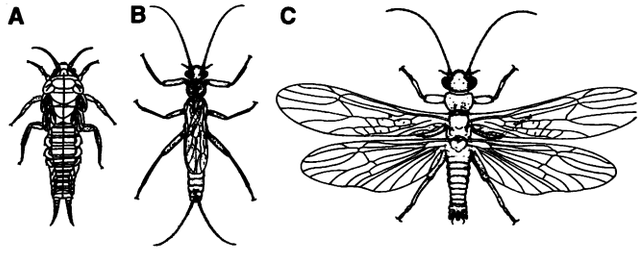

Fig. 2. Variation in wing size and function among fossil and extant stoneflies. (**A**) Fossil Plecopteroid from the lower Permian showing movable gill plates that were probably used for swimming (*4*). (**B**) A flightless short-winged stonefly, *Capnia nana* (*18*). A similar species that we have observed in Pennsylvania (*Allocapnia vivipara*) never flaps its wings under any circumstances but manages to surface skim effectively by raising its wings in response to gusts of wind, thereby sailing across the surface. Large numbers of this species can be observed exiting streams at points where the wind is perpendicular to the direction of current flow. (**C**) *Nemoura flexura* (*18*), a long-winged stonefly that is morphologically similar to *T. burksi*. As for nearly all stoneflies, the literature lacks descriptions of flight or surface-skimming abilities of this species. Drawing (A) is reproduced by permission of John Wiley & Sons from (*19*).

perfamily Nemouroidea near the root of the Plecopteran lineage (*14–16*), where they arose from ancestors whose flight abilities cannot be discerned. It is possible that this lineage has never contained more than marginal fliers, and that extant species such as *T. burksi* have retained an ancient form of locomotion that represents an intermediate stage in the evolution of flight.

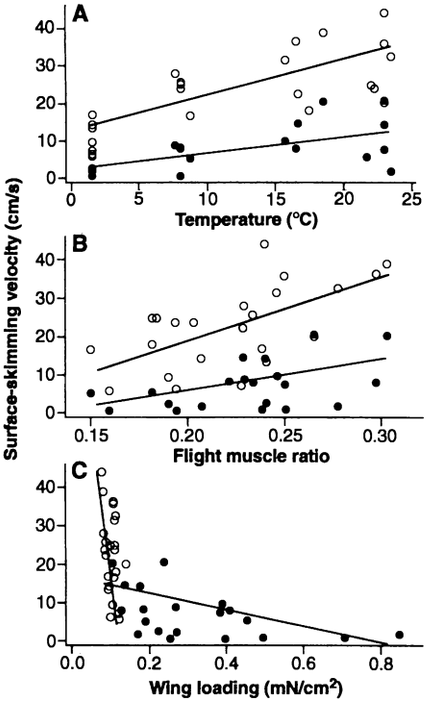

Fig. 3. Variation in surface-skimming velocity as a function of temperature (**A**), flight muscle ratio (**B**), and wing loading (**C**). Open circles represent individuals with unmanipulated wings; closed circles represent the same individuals after the wing area was experimentally reduced by clipping. Univariate regression lines shown on these plots are all significant ($0.001 < P < 0.03$) with the exception of the line for wing loading in normal-winged individuals ($P = 0.11$), which becomes highly significant when temperature and flight muscle ratio effects are considered simultaneously ($P = 0.006$).

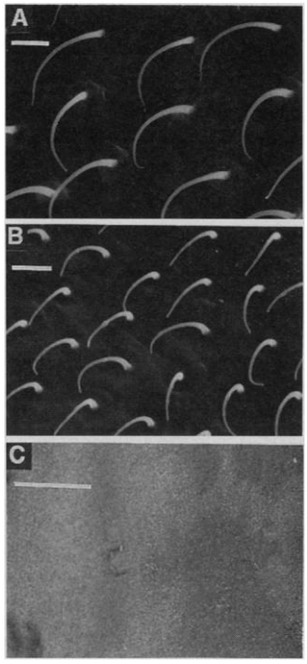

Fig. 5. Scanning electron micrographs showing wet-resistant hairs that uniformly cover the entire wing surface of *T. burksi* stoneflies (**A**) and mayfly subimagos (Ephemeroptera; *Stenonema*) (**B**) but are absent from mayfly imagos [(**C**); same species as in (**B**)]. Scale bars show 10 μm. The importance of resistance to wetting is demonstrated by addition of trace amounts of detergent to the water, which renders *T. burksi* completely incapable of surface skimming.

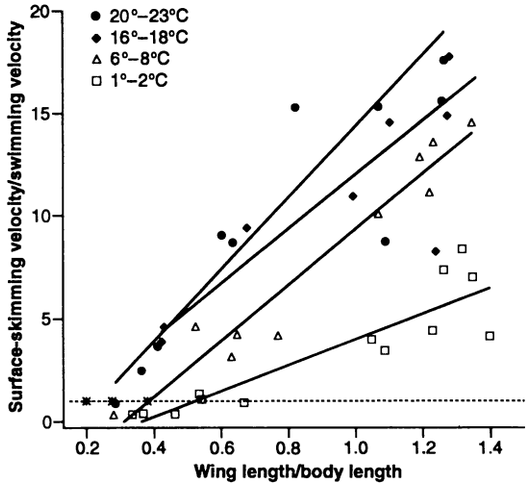

Fig. 4. Ratio of surface-skimming velocity to swimming velocity (mean = 2.39 body lengths per second in stoneflies) as a function of relative wing length. The horizontal line shows the point where surface-skimming performance exceeds swimming performance, that is, where selection should begin to favor elongation of protowings. Asterisks along the horizontal line show the relative wing sizes of fossil Plecopteroid, Ephemeropteroid, and Hemipteroid nymphs from the Paleozoic (*4*). Note that regression lines for all but the lowest temperatures cross the horizontal line within the range of wing sizes that occurred in fossil nymphs. Even at the lowest temperatures (that is, muscle power outputs), with relative wing lengths as low as 0.33, surface skimming allows movement at around 1 body length per second, which may exceed what swimming aquatic nymphs can achieve against the current drag of moving water. Statistics for regression lines are given in (*19*).

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12. Newly emerged *T. burksi* adults were collected along the shoreline of Bald Eagle Creek, Centre County, Pennsylvania, during February and March of 1994 and were stored in the lab at 5°C for up to 3 days before experiments. We measured surface-skimming velocity by dropping individual stoneflies onto water (1-cm depth) in a 24-cm by 34-cm glass dish. Video recordings of surface skimming were made from a camera located vertically above the dish. Three surface-skimming trials were recorded for each wing treatment (before and after wing clipping) for each individual, and from these we selected the straightest, steady-speed regions for measurements of velocity. Three individuals were damaged during wing clipping and were not tested further. The analysis of variance (ANOVA) presented in Table 1 treats data from individuals before and after wing clipping as independent observations, which is appropriate given the lack of correlation of error terms for individuals before and after wing clipping ($r = -0.35$; $P = 0.12$; error terms are residuals from the model shown in Table 1).
13. Body mass of *T. burksi* adults averaged 13.4 mg (SD = 4.6). Insects this small lose heat extremely rapidly and are therefore unable to warm themselves endothermically. Thoracic temperatures of individuals in the field that were walking on the snow surface in full sun did not differ significantly from ambient temperature [mean difference = 0.44°C (SD = 1.09); $P = 0.14$; $n = 15$]. Thus, we assume that thoracic temperatures in the lab also closely approximated ambient temperatures. Citrate synthase activity [V_{max}; a quantitative indicator of maximal rate of tricarboxylic acid cycle activity, that is a limiting factor for adenosine triphosphate (ATP) synthesis] that we measured from *T. burksi* flight muscle homogenates varied with temperature according to the equation V_{max} (micromoles of product/gram of fresh tissue/minute) = −0.98 + 1.22 temperature (°C); $r^2 = 0.99$; $Q_{10} = 1.74$. (where Q_{10} is the increase in reaction rate caused by a 10°C rise in temperature). Lactate dehydrogenase activity (an indicator of anaerobic activity) was nominal at all temperatures. These data show that thermal sensitivity of muscle metabolism in *T. burksi* is indistinguishable from other insects (*11*), that is, their ATP production is not unusually adapted to function at low temperatures, and their muscle power output

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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).
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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).

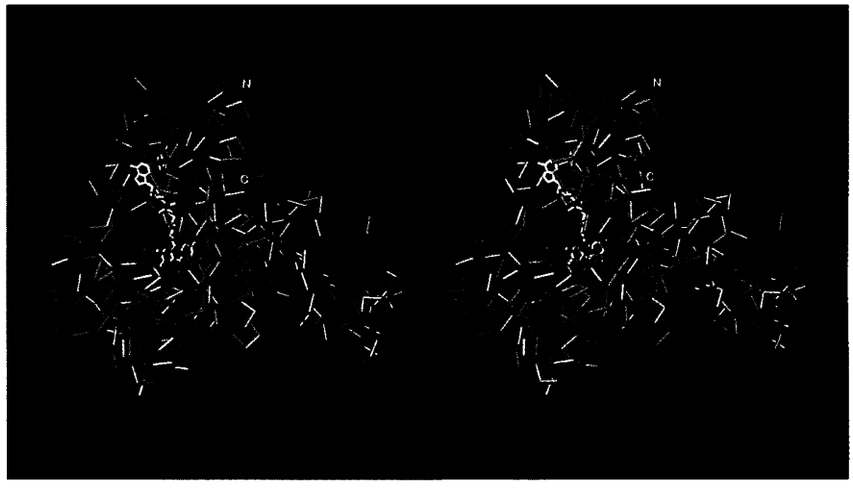

Fig. 1. Stereoview of a C_α tracing of the FCSD molecule with the flavoprotein subunit blue, the cytochrome subunit green, the flavin yellow, and the hemes red. The NH_2- and COOH-termini are labeled in yellow for the flavoprotein and in red for the cytochrome (*21*).

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