with a throat culture positive for group A streptococci ($N = 63$); (ii) all patients with a pneumonic infiltrate visible on chest x-ray ($N = 8$); (iii) all patients with a sore throat severe enough to interfere with normal daily activities and also with a severe cough ($N = 90$); (iv) all patients with a severe cough (associated with a temperature greater than 101°F, or productive of sputum, or of greater than 14 days' duration, or severe enough to interfere with normal daily activities, or with rales on physical examination) ($N = 186$); (v) a randomly selected 30 percent sample of patients with severe sore throat ($N = 46$); and (vi) a randomly selected 10 percent sample of all patients not meeting any of the above criteria ($N = 64$).

6. There were no significant differences among the groups of patients tested for antibodies to each organism with respect to age, sex, socioeconomic status, symptoms, signs, or resolution of symptoms on follow-up contact ($P > 0.05$ by t-test or χ^2 test, two-tailed).

7. B. K. Silverman *et al.*, *Am. J. Dis. Child.* **127**, 498 (1974); I. Ofek *et al.*, *Clin. Pediatr. (Bologna)* **12**, 341 (1973); S. Bergner-Rabinowitz, S. Fleiderman, M. Ferne, K. Rabinowitz, I. Ginsburg, *ibid.* **14**, 804 (1975); A. Elkholy, K. Hafez, R. M. Krause, *Appl. Microbiol.* **27**, 748 (1974); A. Passaglia, I. Fiorini, R. Rossetti, C. M. Avio, *Ann. Sclavo* **20**, 476 (1978).
8. A. M. Behbehani, *Laboratory Diagnosis of Viral, Bedsonial, and Rickettsial Diseases* (Thomas, Springfield, Ill., 1972).
9. R. Taylor, N. R. Somerson, H. C. Turner, R. M. Chanock, *J. Bacteriol.* **85**, 1261 (1963).
10. S.-P. Wang, *J. Clin. Microbiol.* **1**, 250 (1975).
11. Y. M. M. Bishop, S. E. Fienberg, P. W. Holland, *Discrete Multivariate Analysis: Theory and Practice* (MIT Press, Cambridge, Mass., 1975).
12. M. O. Beem and E. M. Saxon, *N. Engl. J. Med.* **296**, 306 (1977).
13. A. L. Komaroff, M. D. Aronson, J. Schachter, *J. Am. Med. Assoc.* **245**, 1319 (1981).
14. K. J. Tack *et al.*, *Lancet* **1980-I**, 116 (1980); T. Marrie *et al.*, *Interscience Conf. Antimicrob. Agents Chemother.* **21**, 357 (1981).
15. J. Schachter and G. Atwood, *J. Am. Vener. Dis. Assoc.* **2**, 12 (1975); C. Watanakunakorn and D. H. Levy, *J. Infect. Dis.* **147**, 364 (1983); C. J. McDonald, W. M. Tierney, S. L. Hui, R. B. Jones, *Clin. Res.* **31**, 302A (1983).
16. J. Schachter, unpublished data.
17. T. C. Quinn *et al.*, *N. Engl. J. Med.* **305**, 195 (1981); K. K. Holmes *et al.*, *ibid.* **292**, 1199 (1975); W. R. Bowie *et al.*, *J. Clin. Invest.* **59**, 735 (1977).
18. B. MacDonald, personal communication.
19. R. Malaty *et al.*, *J. Infect. Dis.* **143**, 853 (1981).
20. W. P. Glezen, W. A. Clyde, R. J. Senior, *J. Am. Med. Assoc.* **202**, 455 (1967).
21. A. S. Evans, V. Allen, S. Sueltmann, *Am. Rev. Respir. Dis.* **96**, 237 (1967); W. J. Mogabgab, *ibid.* **97**, 345 (1968); M. K. Govil *et al.*, *J. Assoc. Physicians India* **23**, 297 (1975).
22. H. L. Moffet, A. C. Siegel, H. K. Doyle, *J. Pediatr.* **73**, 51 (1968).
23. S. S. Gidding *et al.*, *Interscience Conf. Antimicrob. Agents Chemother.* **21**, 355 (1981).
24. G. E. Kenny and H. M. Foy, *N. Engl. J. Med.* **304**, 1240 (1981).
25. G. E. Kenny and R. M. Newton, *ibid.* **225**, 54 (1973); R. Freeman, B. King, M. H. Hambling, *J. Thorac. Cardiovasc. Surg.* **66**, 642 (1973).
26. A. S. Evans and E. C. Diete, *J. Am. Med. Assoc.* **190**, 699 (1964).

27. We thank many colleagues at Brigham and Women's Hospital, Beth Israel Hospital, Harvard Community Health Plan (Kenmore), and Rhode Island Group Health Association for their help with the study; L. Weinstein and E. H. Kass for their advice on the manuscript; and Ortho Diagnostics, Gibco Diagnostics, and Princeton Laboratory Products Company for providing necessary materials. Supported by National Center for Health Services Research grants HS 02063 and HS 04066 and National Eye Institute grant EY 02216 (to J.S.).

18 July 1983; accepted 21 September 1983

Heme-Heme Orientation and Electron Transfer Kinetic Behavior of Multisite Oxidation-Reduction Enzymes

Abstract. *Analysis of the polarized single-crystal absorption spectra of cytochrome cd$_1$ of* Pseudomonas aeruginosa *shows that the heme c and heme d$_1$ groups in each subunit are oriented perpendicularly to each other in both oxidized and reduced forms of the enzyme. These results, together with those of previous kinetic studies, indicate that a perpendicular heme-heme orientation may be an important factor in specifying kinetically slow steps in a sequential series of electron transfer reactions.*

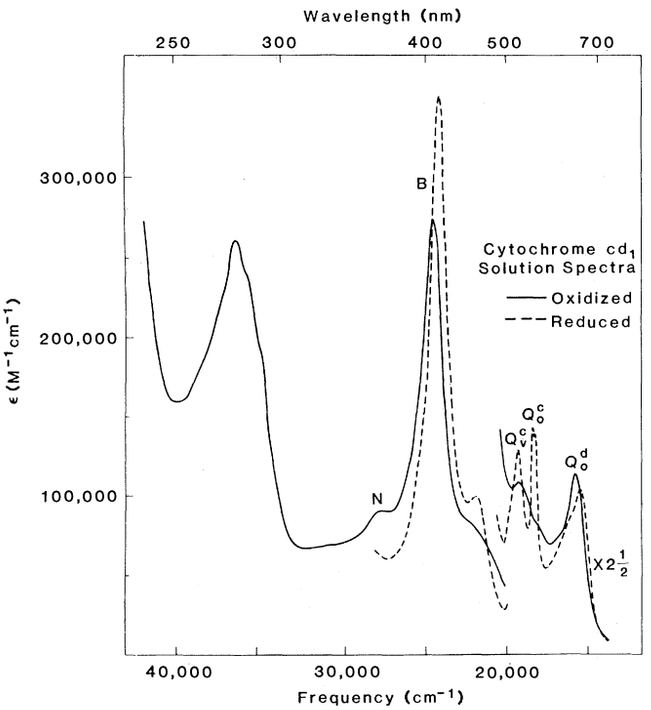
Intramolecular electron transfer is a characteristic feature of the catalytic action of a number of cytochromes containing multiple, prosthetic heme groups (*1–4*). Current theoretical models (*5, 6*) emphasize the importance of heme-heme distance as a controlling factor in such electron transfer processes; however, very little consideration has been given to the role of heme orientation. Spectroscopic (*7–9*) and molecular modeling (*10, 11*) studies suggest that heme groups in multicomponent (and kinetically facile) complexes of cytochromes are aligned in a parallel fashion, and a roughly parallel heme-heme orientation has been found in (diheme) cytochrome c′ through x-ray crystallographic studies (*3*). However, in the tetraheme-containing protein cytochrome c_3, the four heme groups are found in coparallel pairs, each pair being oriented approximately perpendicularly to the other (*4*). We now report, on the basis of polarized single-crystal absorption spectroscopy, that the individual heme c and heme d_1 prosthetic groups in each of the two subunits of nitrite reductase (cytochrome cd$_1$; ferrocytochrome c_{551}: oxygen oxidoreductase, E.C. 1.9.3.3) of *Pseudomonas aeruginosa* are oriented mutually perpendicularly to one another. The implication of this finding, in light of the results of kinetic studies (*12*) of oxidation-reduction reactions of cytochrome cd$_1$, is that heme orientation may be an important factor controlling the rate of electron transfer in multiheme enzymes.

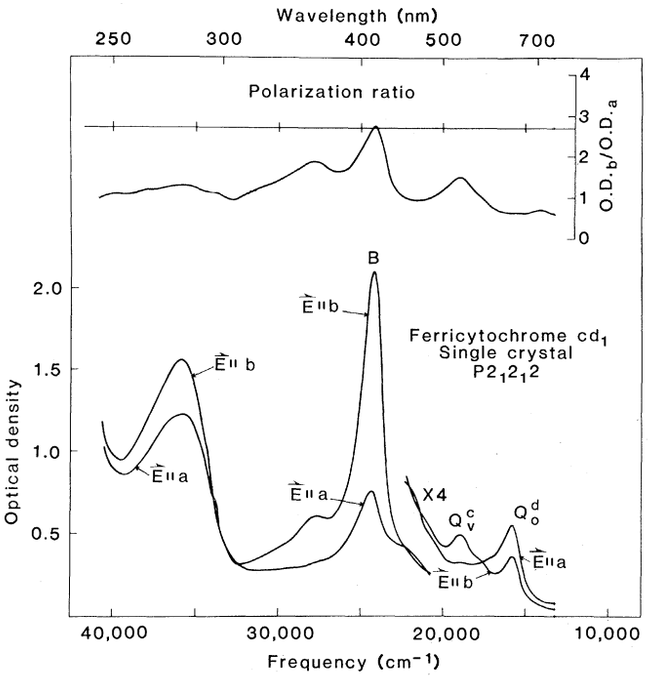
Cytochrome cd$_1$ of *Pseudomonas* is a water-soluble, dimeric protein with two identical subunits of molecular weight 63,000, each with spectroscopically distinct prosthetic groups of heme c (iron protoporphyrin IX) and heme d_1 (iron chlorin) (*13–15*). The enzyme has been crystallized from 70 to 75 percent ammonium sulfate in space group $P2_12_12$ as thin, diamond-shaped plates (*16, 17*). Both heme groups are low-spin ($S = 1/2$) in the oxidized form of the enzyme, whereas the ascorbate-reduced protein contains an ($S = 0$) Fe(II) heme c group and high-spin ($S = 2$) Fe(II) heme d_1 group (*18*). The heme d_1 group is the site of substrate binding and reduction.

The solution absorption spectra of the oxidized and (ascorbate) reduced forms of cytochrome cd$_1$ are illustrated in Fig. 1. Figures 2 and 3 show correspondingly polarized single-crystal absorption spectra. In the crystal spectra the characteristic bands of the heme c chromophore in the visible region are observed primarily in the *b*-crystal spectrum, whereas the absorption bands belonging to the heme d_1 group are observed largely in the orthogonally polarized *a*-crystal spectrum. Thus, except for the strongly overlapping Soret absorption of the heme c and heme d_1 groups, the orthogonally polarized crystal spectra resolve the absorption bands of each of the two types of heme groups in both oxidized and reduced forms of the protein. Comparable observations (data not shown) obtain for crystals of the oxidized enzyme in the presence of 0.2*M* potassium cyanide under which conditions both heme groups are ligated by the cyanide anion (*18*). We have also made similar observations in the case of crystals reduced with sodium dithionite (*19*) or with ascorbate under conditions (0.2*M* potassium cyanide at *p*H 7) in which the cyanide anion is bound only to the heme d_1 group (*18*).

Polarized single-crystal spectroscopic studies of a variety of heme proteins (*20–25*) have established that the heme group exhibits the absorption properties of a square-planar chromophore with respect to the π,π^*–transitions that give rise to the Soret (B) band near 25,000 cm^{-1} and to the Q bands near 17,000 cm^{-1} in both oxidized and reduced states. On this basis, the separate contributions of heme c and heme d_1 to the orthogonally polarized crystal absorption spectra require that the heme c and heme d_1 groups are nearly parallel to the *b* and *a* crystal axes, respectively. Since there is only one pair of spectroscopically distinct heme chromophores in the asymmetric unit of this crystal (*16, 17*), it follows that the heme c and heme d_1 groups are oriented perpendicularly to one another within each subunit of the protein. Coincidence of the molecular dyad with the crystallographic twofold axis specifies further that the heme c group of one subunit is also perpendicular to the heme d_1 group of the neighboring subunit in the dimeric enzyme.

The region of Soret absorption in the spectra of cytochrome cd_1 has overlapping contributions from both heme c and heme d_1 chromophores. Nonetheless, the polarization ratio at the Soret maximum remains unchanged to within ±0.1 unit for both oxidized and reduced forms of the protein. Previous analyses of the polarized single-crystal spectra of heme proteins (*20–25*) have established that the polarization ratio of the Soret band serves as a direct monitor of heme orientation. Therefore, the lack of a detectable change in the polarization ratio upon chemical reduction can mean only that there has been no alteration in the orientation of the two heme groups with respect to the crystal axes. Since comparable observations obtain for the ascorbate reduced form of the enzyme with cyanide bound to the heme d_1 group, it follows that the relative orientation of the two types of heme chromophores in the reduced enzyme is not significantly





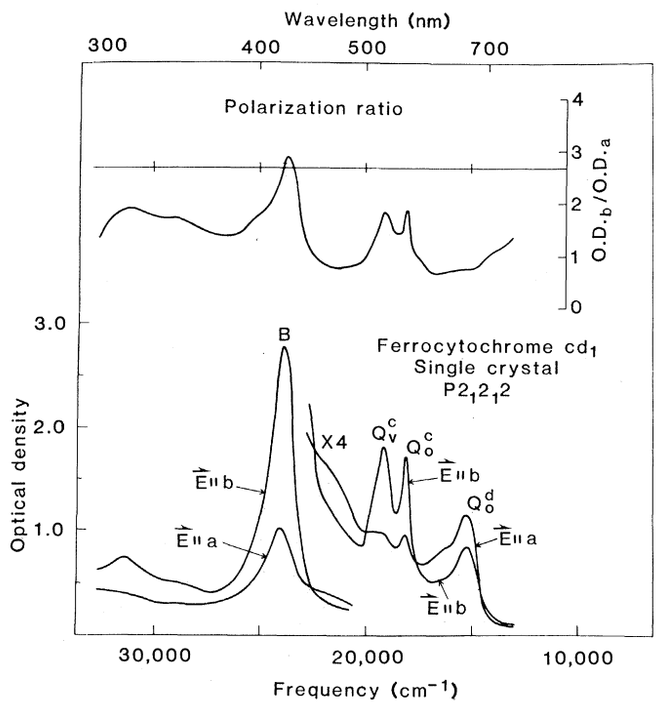


Fig. 1 (upper left). Absorption spectra of cytochrome cd_1 of *P. aeruginosa*. Crystals of the (oxidized) protein prepared in connection with previous studies (*15, 16*) were dissolved in sodium phosphate buffer (μ = 0.2*M*, *p*H 7.0), and the spectra were recorded with a Cary 14 spectrophotometer. The Soret (B) band has overlapping contributions from both heme c and heme d_1, whereas the Q bands of the separate heme c and heme d_1 components are identified on the basis of the known absorption properties of heme c (*27*) and spectra determined for the protein depleted of heme d_1 as well as on the basis of spectra of heme d_1 extracted from the protein with acetone (*15*). (——), Oxidized cytochrome cd_1; (– – –), reduced cytochrome cd_1 obtained by addition of 0.01*M* sodium ascorbate to solutions of the oxidized protein. The extinction coefficients are plotted for the dimeric enzyme on the basis of the protein concentration and a subunit molecular weight of 60,453 (*15*). Fig. 2 (upper right). Polarized single-crystal absorption and polarization ratio spectra of oxidized cytochrome cd_1. Crystals were transferred into 80 to 85 percent saturated ammonium sulfate solutions, buffered to *p*H 7 with 0.01*M* sodium phosphate. The polarized absorption spectra are constructed from data of several crystals of widely different thicknesses, as previously described (*21, 22*). The polarization ratio is plotted as $O.D._b/O.D._a$ in the upper part of the figure as a function of wavelength. For the polarization properties of the crystals used here, the effects of scattered light and crystal misalignment are less than the experimental uncertainty associated with single-crystal optical density and dichroic ratio measurements (*21, 22*). The polarization ratio is estimated with an overall accuracy of ±0.05 unit. The horizontal line indicates the value of the polarization ratio (2.72) that coincides with the position of the Soret maximum for the protein in solution. On the basis of previous polarized single-crystal studies of heme proteins (*20–26*), this value corresponds closely to the ratio of the integrated polarized crystal Soret bands and serves as a direct monitor of heme orientation. Fig. 3 (lower left). Polarized single-crystal absorption and polarization ratio spectra of ascorbate-reduced cytochrome cd_1. Crystals of the oxidized protein transferred to buffered ammonium sulfate solutions as in Fig. 2 were equilibrated with 0.01*M* sodium ascorbate. Reduction of the protein was complete within 45 minutes, and no further spectral change was observed on preserving the reduced crystals under anaerobic conditions for several days. The value of the polarization ratio at 419 nm corresponding to the Soret maximum is 2.68. Other conditions were as described in Fig. 2.

changed further upon ligand binding.

Our work, together with earlier observations on cytochromes c, c′, and c_3, suggests a possible role for heme orientation in regulating electron transfer rates in biological systems. The results of these spectroscopic studies show that the heme groups retain their mutually perpendicular orientation in both oxidized and reduced forms. Intramolecular electron transfer of heme c to heme d_1 in cytochrome cd_1 is relatively sluggish in the absence of dioxygen ($k \simeq 0.3$ sec^{-1} at 25°C) (*12, 15*), in contrast to rates observed for kinetically facile complexes of cytochromes. Since the average interheme distance in cytochrome cd_1 (*12, 15*) is comparable to that in kinetically facile multiheme oxidation-reduction enzymes (*2–11*), the perpendicular disposition of the heme c and heme d_1 groups is logically responsible for the slow rate of electron transfer.

We have previously shown that the orbitals associated with porphyrin-to-metal and metal-to-porphyrin charge-transfer transitions are identical to those involved in oxidation-reduction reactions (*20, 26*). In general, the probability of charge-transfer transitions polarized perpendicularly to the heme plane is small in comparison to that for charge-transfer transitions polarized in the heme plane (*20*). In oxidation-reduction reactions the probability of the electron transfer event is determined by the extent of orbital overlap between the donor and acceptor molecules according to a quantum mechanical description (*5, 6*) that is analogous to that for electronic transitions to excited states. Thus, the perpendicular orientation of the heme c and heme d_1 groups in cytochrome cd_1 may also predispose to a small transition probability (that is, small rate constant) for the redox process because of similar disposition of the orbitals of the porphyrin ring and the iron involved in the oxidation-reduction process.

The physiological function of cytochrome c_3 with four hemes arranged in approximately perpendicular pairs appears to be the storage of reducing equivalents (*4*); similarly, on the basis of kinetic studies of electron transfer reactions of cytochrome cd_1 (*12, 15*), there is now a strong hint that the ability of this multiheme enzyme to store electrons prior to intramolecular reduction steps may be enhanced by a perpendicular heme-heme disposition. In contrast, cytochrome complexes characterized by kinetically facile intermolecular electron transfer appear to have mutually parallel heme orientations (*3, 7–11*). This correlation suggests that electron transfer rates in multiheme enzyme complexes are strong functions of both orientation and distance and that a perpendicular heme-heme orientation may be an important factor in specifying kinetically slow steps in a sequential series of electron transfer reactions.

MARVIN W. MAKINEN
STEVEN A. SCHICHMAN
SUSAN C. HILL
Department of Biophysics and Theoretical Biology, University of Chicago, Cummings Life Science Center, Chicago, Illinois 60637
HARRY B. GRAY
Arthur Amos Noyes Laboratory, California Institute of Technology, Pasadena 91125

References and Notes

1. R. Lemberg and J. Barrett, *Cytochromes* (Academic Press, New York, 1973), chap. 6, pp. 217–326.
2. S. D. Fuller, R. A. Capaldi, R. Henderson, *J. Mol. Biol.* **134**, 305 (1979).
3. P. C. Weber *et al.*, *Nature (London)* **286**, 302 (1980).
4. R. Haser *et al.*, *ibid.* **282**, 806 (1979); Y. Higuchi *et al.*, *J. Biochem. (Tokyo)* **89**, 1659 (1981); T. Yagi, H. Inokuchi, K. Kimura, *Accts. Chem. Res.* **16**, 2 (1983).
5. J. J. Hopfield, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3640 (1974).
6. J. Jortner, *J. Chem. Phys.* **64**, 4860 (1976).
7. J. M. Vanderkooi, R. Landesberg, G. W. Hayden, C. S. Owen, *Eur. J. Biochem.* **81**, 339 (1977).
8. J. S. Leigh and J. J. Harmon, *Biophys. J.* **17**, 251a (1977).
9. M. Erecinska, D. F. Wilson, J. K. Blaisie, *Biochim. Biophys. Acta* **501**, 63 (1978).
10. T. L. Poulos and J. Kraut, *J. Biol. Chem.* **255**, 10322 (1980); T. L. Poulos and A. G. Mauk, *ibid.* **258**, 7369 (1983).
11. F. R. Salemme, *J. Mol. Biol.* **102**, 563 (1976).
12. S. A. Schichman and H. B. Gray, *J. Am. Chem. Soc.* **103**, 7794 (1981); in preparation.
13. T. Kuronen and N. Ellfolk, *Biochim. Biophys. Acta* **275**, 308 (1972); T. Kuronen, M. Saraste, N. Ellfolk, *ibid.* **393**, 48 (1975).
14. J. C. Gudat, J. Singh, D. C. Wharton, *ibid.* **292**, 376 (1973).
15. S. A. Schichman, thesis, University of Chicago (1982).
16. T. Takano, R. E. Dickerson, S. A. Schichman, T. E. Meyer, *J. Mol. Biol.* **133**, 185 (1979).
17. C. W. Akey, K. Moffat, D. C. Wharton, S. J. Edelstein, *ibid.* **136**, 19 (1980).
18. T. A. Walsh, M. K. Johnson, C. Greenwood, D. Barber, J. P. Springall, A. J. Thomson, *Biochem. J.* **177**, 29 (1979).
19. Akey *et al.* (*17*) report that reduction with solid sodium dithionite leads to rapid cracking and complete disintegration of crystals. We are unable to explain the origin of their observations since the crystals employed in this study, obtained from two different and highly purified preparations of the protein (*15*), were stable for several days upon reduction. Furthermore, no change in or loss of dichroism of the reduced crystals was observed after several days when crystals reduced with either ascorbate or sodium dithionite were maintained under totally anaerobic conditions.
20. M. W. Makinen and A. K. Churg, in *Physical Bioinorganic Chemistry Series. Iron-Porphyrins*, A. B. P. Lever and H. B. Gray, Eds. (Addison-Wesley, Reading, Mass., 1983), vol. 1, pp. 143–235.
21. A. K. Churg and M. W. Makinen, *J. Chem. Phys.* **68**, 1913 (1978); see *erratum*, *ibid.* **69**, 2268 (1978).
22. M. W. Makinen, A. K. Churg, H. A. Glick, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2291 (1978).
23. W. A. Eaton and R. W. Hochstrasser, *J. Chem. Phys.* **46**, 2533 (1967).
24. M. W. Makinen and W. A. Eaton, *Nature (London)* **247**, 62 (1973); *Ann. N.Y. Acad. Sci.* **206**, 210 (1973).
25. W. A. Eaton and R. M. Hochstrasser, *J. Chem. Phys.* **49**, 985 (1968).
26. M. W. Makinen, A. K. Churg, Y. Y. Shen, S. C. Hill, in *Electron Transport and Oxygen Utilization*, H. Chien *et al.*, Eds. (Elsevier, New York, 1982), p. 101–109.
27. E. Margoliash and N. Frohwirt, *Biochem. J.* **71**, 570 (1959).
28. We thank Dr. T. Takano for providing crystals of cytochrome cd_1. Supported by NSF grant PCM 77-13479 (M.W.M.), American Heart Association grant-in-aid 77378 (M.W.M.), NSF grant CHE 82-18502 (H.B.G.), and NIH training grant 1-T32-HD-07009 (S.A.S.). This is contribution No. 6774 from the Arthur Amos Noyes Laboratory.

13 June 1983; accepted 21 September 198[illegible]

Erythrocyte Form of Spectrin in Cerebellum: Appearance at a Specific Stage in the Terminal Differentiation of Neurons

Abstract. *The developing chicken cerebellum contains two forms of the plasma membrane–associated actin-binding protein spectrin. The brain form, αγ-spectrin (fodrin), is expressed constitutively in all neuronal cell bodies and processes during all stages of cerebellar morphogenesis. On the other hand, the erythrocyte form, αβ′β-spectrin, accumulates exclusively at the plasma membrane of the cell bodies of Purkinje and granule cells and of neurons in cerebellar nuclei, but only after these cells have become postmitotic and have completed their migration to their final positions in the cerebellum. The appearance of αβ′β-spectrin coincides temporally with the establishment of axosomatic contacts on these three neuronal cell types, which suggests that αβ′β-spectrin accumulates in response to the formation of functional synaptic connections during cerebellar ontogeny.*

The morphogenesis of the major neuronal cell types of the cerebellum has been extensively studied by means of light and electron microscopy (*1*). In general, cells undergo an initial proliferative phase, and then a postmitotic phase during which each cell type migrates to a specific area of the cortex. Once the cell reaches its designated position, it undergoes terminal differentiation upon the establishment of synaptic connections with other neurons. Thus, the cerebellum provides an ideal system to study the development of the chemical and structural heterogeneity of synapses during neuronal morphogenesis.