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LETTERS TO THE EDITOR

Crystallographic Characterization of Flavodoxin from *Anacystis nidulans*

Flavodoxin isolated from the blue-green alga, *Anacystis nidulans*, crystallizes from ammonium sulfate in space group $P2_12_12_1$, with $a = 57{\cdot}08$ Å, $b = 69{\cdot}24$ Å and $c = 45{\cdot}55$ Å. The diffraction patterns extend to a resolution of at least 1·8 Å. Reduction of the flavin mononucleotide in the crystalline protein, to either the semi-quinone or fully reduced (hydroquinone) state, results in minimal changes in cell dimensions and diffracted intensities. The higher molecular weight (19,000 to 20,000) and spectral properties of the *A. nidulans* protein, along with the near-isomorphism of crystals of the three oxidation states, distinguish this crystalline flavodoxin from the corresponding proteins of *Clostridium MP* and *Desulfovibrio vulgaris*, whose three-dimensional structures are known. In contrast to *Clostridium* flavodoxins, but like the *D. vulgaris* protein, *A. nidulans* flavodoxin is capable of binding riboflavin in place of flavin mononucleotide ($K_a = 2 \times 10^6$ M^{-1}).

The flavodoxins are a group of microbial proteins that supplant or supplement plant and bacterial ferredoxins in a variety of electron transfer reactions. The first of these flavin mononucleotide-containing proteins to be purified was isolated from the blue-green alga, *Anacystis nidulans*, by Smillie (1963,1965) who showed that it could replace ferredoxin in the light-dependent reduction of $NADP^+$ by chloroplasts. Subsequently flavodoxins have been found in at least a dozen organisms (Yoch & Valentine, 1972) and a number of physical and chemical studies of these proteins has been reported, including the determination of high-resolution X-ray structures of flavodoxins from *Clostridium MP* (Andersen *et al.*, 1972; Burnett *et al.*, 1974) and *Desulfovibrio vulgaris* (Watenpaugh *et al.*, 1972,1973). In this Letter we present preliminary crystallographic data on flavodoxin from *A. nidulans* (sometimes called phytoflavin), and consider some of the distinctive properties of this flavodoxin which suggest that structure analysis may provide results of significance for the biochemistry of flavoproteins.

Purification of the protein has been described earlier (Smillie, 1963,1965; Smillie & Entsch, 1971; Bothe *et al.*, 1971). We have crystallized *A. nidulans* flavodoxin† from ammonium sulfate (2·3 to 2·5 M) or from Na/K phosphate at pH 6·8. The crystals are prisms elongated in the *a* direction, and tend to cluster in rosettes. However, by slow growth from solutions of the semiquinone form, we can obtain crystals of adequate thickness for diffraction studies. The *mmm* diffraction symmetry and systematic absences along all axes lead to assignment of space group $P2_12_12_1$; the cell dimensions measured on a diffractometer are: $a = 57{\cdot}08$ Å, $b = 69{\cdot}24$ Å, and $c = 45{\cdot}55$ Å. A photograph of the *hk*0 zone, showing reflections corresponding to Bragg spacings as small as 1·83 Å, is reproduced in Plate I. The crystal density has not been determined, but assuming a molecular weight of 20,000 (see below), the volume per dalton is 2·25 Å^3 for one molecule per asymmetric unit. With $\bar{v} = 0{\cdot}75$, two such molecules

† *Anacystis nidulans* was grown for us on a large scale at Argonne National Laboratory.

per asymmetric unit would exceed the volume of the unit cell. Even if the molecular weight and $\bar{v}$ were 16,000 and 0·70, respectively, the volume fraction of solvent for two molecules per asymmetric unit would be only 17·4%, a highly improbable value (Matthews, 1968).

Intensities from oxidized crystals have been measured to a resolution of 2·0 Å. We intend to use *A. nidulans* flavodoxin to test the application of vector search methods (Nordman, 1972) to protein structure analysis, employing vector sets generated from atomic positions common to both *Clostridium MP* and *D. vulgaris* flavodoxins. At the same time we are undertaking the preparation of heavy-atom derivatives. Sm^{3+} was expected to provide a derivative, as it had been used in determination of the other flavodoxin structures (Andersen *et al.*, 1972; Watenpaugh *et al.*, 1972). To our dismay, Sm^{3+} and the other lanthanides tested dissolved crystals of *A. nidulans* flavodoxin. Alternative approaches to the attachment of heavy atoms, including the use of cross-linked crystals (Quiocho & Richards, 1964), are being explored.

The molecular weight of *A. nidulans* flavodoxin has been the subject of some disagreement. Smillie & Entsch (1971) suggested a value of approximately 20,000 from amino acid analysis, gel filtration, and flavin mononucleotide content, whereas Bothe *et al.* (1971) arrived at a molecular weight of 16,000 from sedimentation and diffusion measurements. We have made another estimate of the molecular weight by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber *et al.*, 1972). The molecular weight determined by this technique, using *Clostridium MP* flavodoxin, myoglobin, trypsin and chymotrypsinogen as standards, was between 19,000 and 19,500. Assuming the validity of the higher molecular weight estimates, the chain of *A. nidulans* flavodoxin will include 25 to 30 more residues than *Clostridium MP* flavodoxin. Several other flavodoxins possess polypeptide chains significantly longer than those of *Clostridium MP* (138 residues) or *D. vulgaris* (148 residues) (Fox *et al.*, 1972). In view of the similarity of the folding of flavodoxins and several pyridine nucleotide dehydrogenases (Rossmann *et al.*, 1974), it will be interesting to determine the location of the additional residues in the higher molecular weight species. Although the extra amino acids may be distributed along the chain, it is conceivable that a new segment of secondary structure occurs in the larger flavodoxins†.

Flavodoxins have been classified according to their ability to bind flavin analogs. Both *Peptostreptococcus elsdenii* and *Clostridium MP* flavodoxins fail to bind flavins unless the phosphate group is present (Mayhew, 1971*b*), but *D. vulgaris*, *A. vinelandii*, and certain other flavodoxins interact strongly with riboflavin ($K_a \cong 10^6$ M^{-1}). *A. nidulans* flavodoxin belongs to the latter family of flavodoxins (Fig. 1). Perturbations of the riboflavin spectrum, which result from binding to apoflavodoxin, have been utilized to calculate a K_a for riboflavin of $2{\cdot}0 \pm 0{\cdot}5 \times 10^6$ M^{-1}. *A. nidulans* flavodoxin thus affords us the opportunity to compare the structures of the riboflavin protein and the flavin mononucleotide protein.

Prior to the determination of the structures of *D. vulgaris* and *Clostridium MP* flavodoxins, dissimilarities in the optical and circular dichroism spectra of flavodoxins derived from different organisms (Edmondson & Tollin, 1971; D'Anna & Tollin, 1972) had suggested that the arrangement of the active center of flavodoxins was not absolutely conserved during evolution. Comparison of the three-dimensional structures of *D. vulgaris* and *Clostridium MP* flavodoxins has verified this interpreta-

† Dr K. T. Yasunobu and co-workers at the University of Hawaii have begun amino acid sequence analysis of *A. nidulans* flavodoxin.

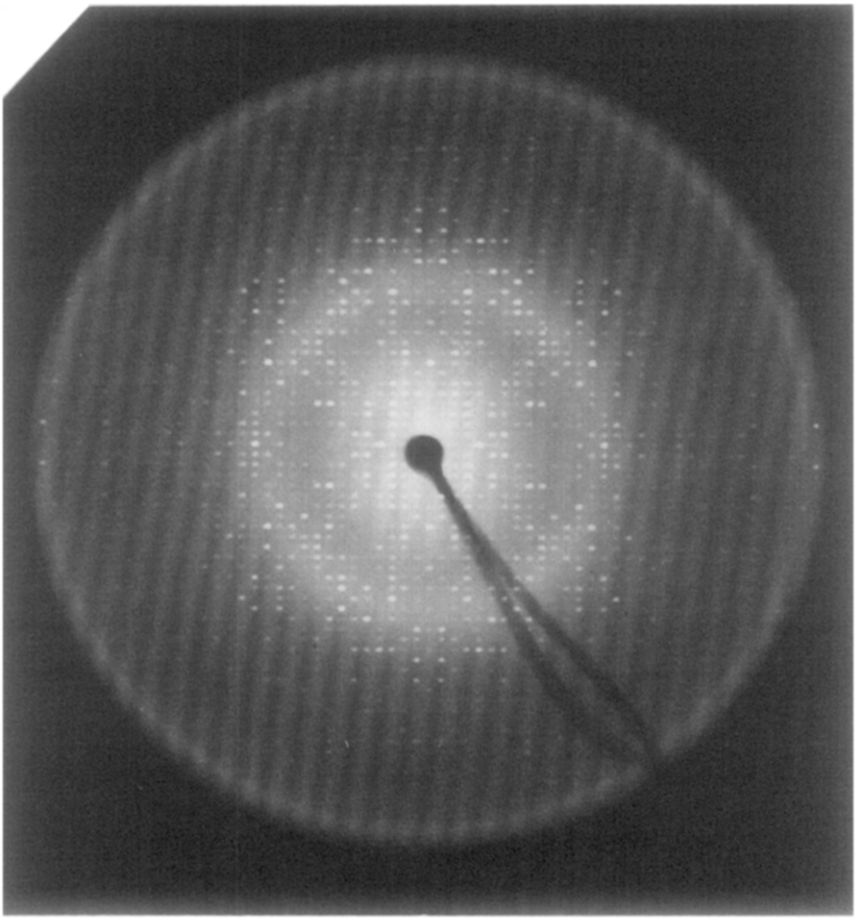

PLATE I. A precession photograph of the *hk*0 zone; precession angle = 25°. The crystal of *nidulans* flavodoxin was mounted in a quartz capillary with the a axis parallel to the spindle.

[*facing p. 124*

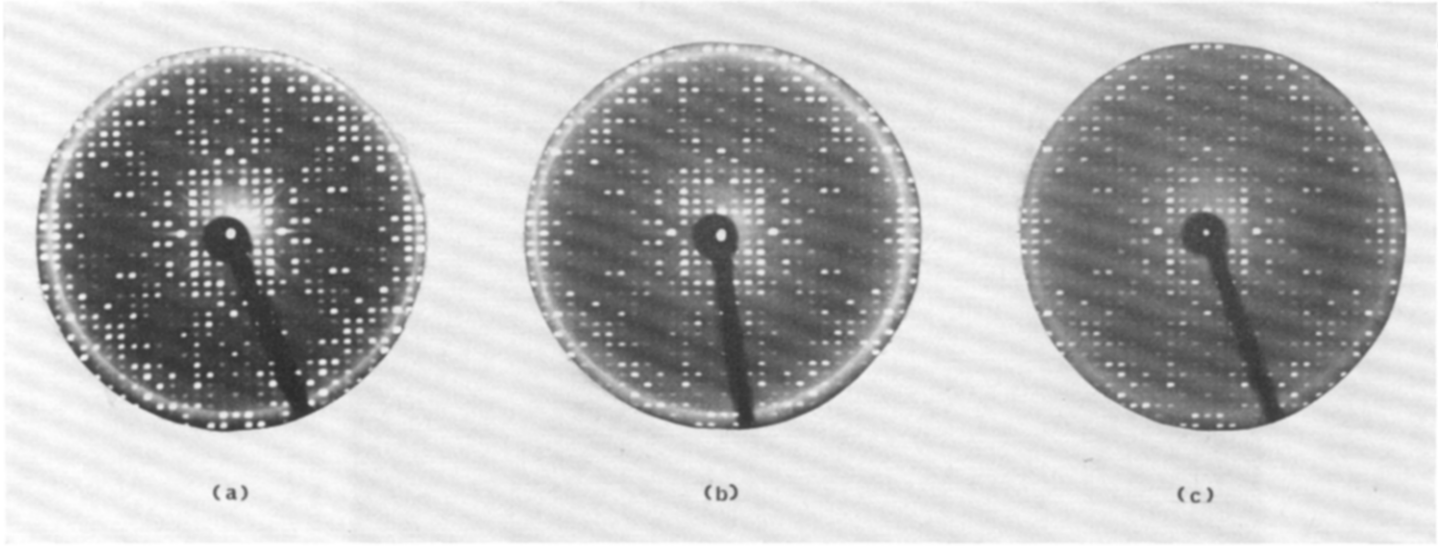

PLATE II. Precession photographs of crystals of *A. nidulans* flavodoxin in each of the three oxidation states: (a) oxidized, (b) semiquinone, (c) reduced. Precession angle = 12°. The crystals were grown from solutions of the semiquinone form, and either oxidized by exposure to air, or reduced at pH 7·2 by addition of excess dithionite. The predominant oxidation state of the molecules in the crystals photographed for this Plate could be ascertained by visual examination under a microscope at low resolution, since in transmitted light oxidized crystals appear orange, semiquinone, purple; and hydroquinone, almost colorless (Smillie & Entsch, 1971; Entsch & Smillie, 1972).

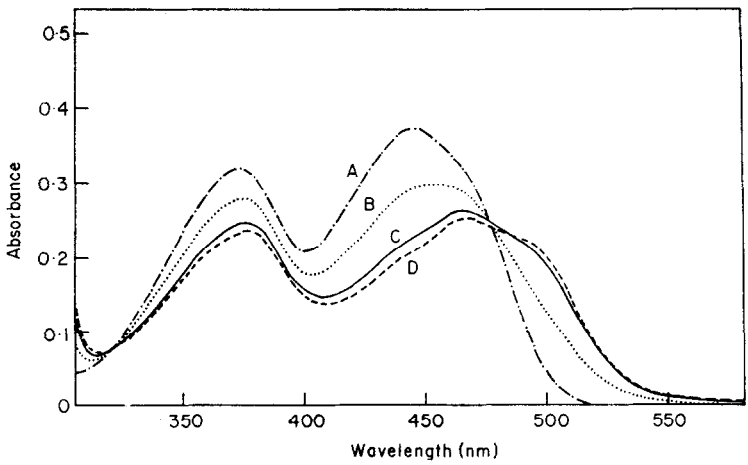

FIG. 1. Changes in the absorption spectrum of riboflavin upon addition of *A. nidulans* apoprotein. Portions of a solution of 1·0 mM-apoflavodoxin in 0·20 M-potassium phosphate buffer, pH 7·3, containing 1 mM-EDTA were added to 1·20 ml of a solution of riboflavin in 50 mM-phosphate, 30 mM-EDTA. Curve A, 30·5 mM-riboflavin; curves B, C and D, spectra after addition of 20, 40 and 60 μl of apoprotein. For curve D, 97% of the riboflavin is complexed with protein. The temperature was 25°C.

tion; different amino acid residues surround the isoalloxazine ring in these two flavodoxins (Burnett *et al.*, 1974; Watenpaugh *et al.*, 1973). The visible spectrum of oxidized *A. nidulans* flavodoxin cannot be superimposed on the spectra of either *D. vulgaris* or *Clostridium MP* flavodoxins. In the *A. nidulans* protein, the long-wavelength absorption maximum appears at 466 nm, with $\varepsilon = 9000\ \text{M}^{-1}\ \text{cm}^{-1}$ (Smillie & Entsch, 1971); the corresponding maxima for *Clostridium MP* and *D. vulgaris* flavodoxins occur at 445 nm and 456 nm, respectively, with larger molar extinctions (Mayhew, 1971*a*; Dubourdieu & LeGall, 1970). Furthermore, the spectrum of the fully reduced (hydroquinone) form of *A. nidulans* flavodoxin is distinctive. The lowest energy absorption maximum appears at shorter wavelengths in *A. nidulans* (λ_{max} = 360 nm) than in *Clostridium MP* flavodoxin (λ_{max} = 367 nm) and the pronounced shoulder, which occurs at 450 nm in the spectrum of reduced *Clostridium MP* flavodoxin, is absent from the spectrum of reduced *A. nidulans* flavodoxin (Ghisla *et al.*, 1974; Entsch & Smillie, 1972). These spectral differences lead us to anticipate that the flavin environment in *A. nidulans* flavodoxin may be unlike that in either of the known structures.

In crystals of *Clostridium MP* flavodoxin, one-electron reduction of the protein-bound flavin mononucleotide is accompanied by large changes in the diffracted intensities (Ludwig *et al.*, 1969), although nuclear magnetic resonance spectroscopy and X-ray structure analysis both demonstrate that the conformation changes resulting from reduction are small (Ludwig *et al.*, 1974; James *et al.*, 1973). Moderate-resolution diffraction patterns of crystals of *A. nidulans* flavodoxin, on the other hand, show little if any change as the flavin is progressively reduced (Plate II). Comparison of the three oxidation states, which may suggest how combination with apoflavodoxin produces shifts in the redox potentials of flavin mononucleotide (Draper & Ingraham, 1968; Mayhew, 1971*a*; Van Lin & Bothe, 1972), should be enormously simplified by the close isomorphism.

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REFERENCES

Andersen, R. D., Apgar, P. A., Burnett, R. M., Darling, G. D., LeQuesne, M. E., Mayhew, S. G. & Ludwig, M. L. (1972). *Proc. Nat. Acad. Sci., U.S.A.* **69**, 3189–3191.

Bothe, H., Hemmerich, P. & Sund, H. (1971). In *Flavins and Flavoproteins* (Kamin, H., ed.), pp. 211–226, University Park Press, Baltimore.

Burnett, R. M., Darling, G. D., Kendall, D. S., LeQuesne, M. E., Mayhew, S. G., Smith, W. W. & Ludwig, M. L. (1974). *J. Biol. Chem.* **249**, 4383–4392.

D'Anna, J. A. & Tollin, G. (1972). *Biochemistry*, **11**, 1073–1080.

Draper, R. D. & Ingraham, L. L. (1968). *Arch. Biochem. Biophys.* **125**, 802–808.

Dubourdieu, M. & LeGall, J. (1970). *Biochem. Biophys. Res. Commun.* **38**, 965–972.

Edmondson, D. E. & Tollin, G. (1971). *Biochemistry*, **10**, 113–124.

Entsch, B. & Smillie, R. M. (1972). *Arch. Biochem. Biophys.* **151**, 378–386.

Fox, J. L., Smith, S. S. & Brown, J. R. (1972). *Z. Naturforsch.* **27b**, 1096–1100.

Ghisla, S., Massey, V., Lhoste, J.-M. & Mayhew, S. G. (1974). *Biochemistry*, **13**, 589–597.

James, T. L., Ludwig, M. L. & Cohn, M. (1973). *Proc. Nat. Acad. Sci., U.S.A.* **70**, 3292–3295.

Ludwig, M. L., Andersen, R. D., Mayhew, S. G. & Massey, V. (1969). *J. Biol. Chem.* **244**, 6047–6048.

Ludwig, M. L., Burnett, R. M., Darling, G. D., Jordan, S. R., Kendall, D. S. & Smith, W. W. (1975). In *4th Steenbock Symposium*, Madison Wisc. (Sundaralingam, M. & Rao, S. T., eds), in the press,

Matthews, B. (1968). *J. Mol. Biol.* **33**, 491–497.

Mayhew, S. G. (1971*a*). *Biochim. Biophys. Acta*, **235**, 276–288.

Mayhew, S. G. (1971*b*). *Biochim. Biophys. Acta*, **235**, 289–302.

Nordman, C. E. (1972). *Acta Crystallogr. Sect. A*, **28**, 134–143.

Quiocho, F. & Richards, F. M. (1964). *Proc. Nat. Acad. Sci., U.S.A.* **52**, 833–839.

Rossmann, M. G., Moras, D. & Olsen, K. W. (1974). *Nature (London)*, **250**, 194–199.

Smillie, R. M. (1963). *Plant Physiology Suppl.* **38**, XXVIII–XXIX.

Smillie, R. M. (1965). *Biochem. Biophys. Res. Commun.* **20**, 621–629.

Smillie, R. M. & Entsch, B. (1971). *Methods in Enzymology*, **23**, 504–514.

Van Lin, B. & Bothe, H. (1972). *Arch. Microbiol.* **82**, 155–172.

Watenpaugh, K. D., Sieker, L. C., Jensen, L. H., LeGall, J. & Dubourdieu, M. (1972). *Proc. Nat. Acad. Sci., U.S.A.* **69**, 3185–3188.

Watenpaugh, K. D., Sieker, L. C. & Jensen, L. H. (1973). *Proc. Nat. Acad. Sci., U.S.A.* **70**, 3857–3860.

Weber, K., Pringle, J. R. & Osborn, M. (1972). *Methods in Enzymology*, **26**, 3–27.

Yoch, D. C. & Valentine, R. C. (1972). *Annu. Rev. Microbiol.* **26**, 139–157.