spectral regions, it is impossible to verify this speculation by optical rotatory dispersion or circular dichroism measurements.

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References

Auborn, J. J., Eyring, E. M., and Choules, G. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1966.
Bowen, T. J. (1970), An Introduction to Ultracentrifugation, New York, N. Y., Wiley-Interscience.
Cannon, M. R., and Fenske, M. R. (1944), *Ind. Eng. Chem., Anal. Ed. 16*, 55.
Chervenka, C. H. (1970), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Beckman Instruments, Inc.
Choules, G. L., and Bjorklund, R. F. (1970), *Biochemistry 9*, 4759.
Choules, G. L., and Gray, W. R. (1971), *Biochem. Biophys. Res. Commun. 45*, 849.
Fox, C. F. (1972), *Sci. Amer. 226*, 30.
Ji, T. H., Hess, J. L., and Benson, A. A. (1968), *in* Comparative Biochemistry and Biophysics of Photosynthesis, Shibuta, K., Takamiya, A., Jagendorf, A. T., and Fuller, R. C., Ed., Tokyo University Press, p 36.
Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.
Marchesi, V. T., and Andrews, E. P. (1971), *Science 174*, 1247.
Melchior, P. L., Morowitz, H. J., Sturtevant, J. M., and Tsong, T. Y. (1970), *Biochim. Biophys. Acta 219*, 114.
Reynolds, J. A., and Tanford, C. (1970a), *Proc. Nat. Acad. Sci. 66*, 1002.
Reynolds, J. A., and Tanford, C. (1970b), *J. Biol. Chem. 245*, 5161.
Robertson, J. D. (1964), *in* Cellular Membranes in Development, Locke, M., Ed., New York, N. Y., Academic Press, p 1.
Robinson, D. R., and Jencks, W. P. (1965), *J. Amer. Chem. Soc. 87*, 2470.
Singer, S. J., and Nicolson, G. L. (1972), *Science 175*, 718.
Sober, H. A., Ed. (1968), Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co., pp C-10–C-23.
Sober, H. A., Ed. (1970), Handbook of Biochemistry, 2nd ed, Cleveland, Ohio, Chemical Rubber Co.
Steim, J. M., Tourtellotte, M. E., Reinert, J. C., McElhaney, R. N., and Rader, R. L. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 104.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 394.
Townend, R., Herskovits, T. T., Timasheff, S. N., and Gorbunoff, M. J. (1969), *Arch. Biochem. Biophys. 129*, 567.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
Vanderkooi, G., and Green, D. E. (1971), *Bioscience 21*, 409.

Flavine–Protein Interactions in Flavoenzymes. Effect of Chemical Modification of Tryptophan Residues upon Flavine Mononucleotide Binding and Protein Fluorescence in *Azotobacter* Flavodoxin†

Jack Ryan and Gordon Tollin*

ABSTRACT: *N*-Bromosuccinimide easily oxidizes two of the four tryptophan side chains of *Azotobacter vinelandii* flavodoxin. The reaction occurs more rapidly and to a somewhat greater extent with the apoprotein than with the holoprotein. The titration follows a sigmoidal curve and the initial lag phase is essentially eliminated by blocking the single sulfhydryl group of the protein *via* reaction with dithiobis(nitrobenzoic acid). Oxidation of the tryptophans is associated with a loss of flavine mononucleotide (FMN) binding ability. Analysis of these results indicates that a single tryptophan residue is involved in binding. Titration of the decrease in tryptophan fluorescence of the apoprotein upon *N*-bromosuccinimide oxidation demonstrates that a single tryptophan is responsible for approximately 90% of the emission intensity. This correlates with the quenching of tryptophan fluorescence upon FMN binding to the native apoprotein. Oxidation of the tryptophans completely eliminates the near-uv circular dichroism bands and produces changes in the far-uv spectra which suggest conformational alterations. Blockage of the sulfhydryl group with dithiobis(nitrobenzoic acid) has no effect on FMN binding, but allows dithionite to reduce the bound FMN at a much faster rate to form the semiquinone.

Previous work from this laboratory (D'Anna and Tollin, 1971) has shown that approximately 90% of the tryptophan fluorescence of *Azotobacter* apoflavodoxin is quenched upon FMN binding. The quenching follows second-order kinetics with a rate constant identical with that for the flavine fluorescence quenching which also occurs upon binding. Similar observations have been made with a variety of other flavodoxins (D'Anna and Tollin, 1972; Mayhew, 1971), and thus this appears to be a general property of this group of flavoproteins. In view of these results and the recent demonstration, based on X-ray crystal structure determination, that a tryptophan side chain is in close proximity to the isoalloxazine ring of

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the bound FMN in the *Desulfovibrio vulgaris* flavodoxin (Watenpaugh *et al.*, 1973), we have carried out a study of the chemical modification of tryptophan side chains in the *Azotobacter* flavodoxin. McCormick (1970) has reported that two out of the four tryptophyl residues in *Clostridium pasteurianum* flavodoxin can be oxidized by *N*-bromosuccinimide and that this is accompanied by FMN release and loss of activity.

Experimental Section

Materials

Azotobacter vinelandii flavodoxin was isolated by the method of Hinkson and Bulen (1967). Apoprotein was prepared by the procedure of Edmondson and Tollin (1971a). Trypsin (two-times crystallized) was obtained from Worthington Biochemical Corp. *N*-Bromosuccinimide was from J. T. Baker Co. Dithiobis(nitrobenzoic acid) (Nbs_2)[1] was from Calbiochem. Sodium dithionite (90% practical) was from Eastman Organic Chemicals. FMN was obtained from Calbiochem and purified on DEAE-cellulose.

Methods

Tryptophan residues in flavodoxin (pH 6–7) or trypsin (pH 4) were oxidized by aliquots of *N*-bromosuccinimide as described by Spande and Witkop (1967). Changes in optical density were measured on the Gilford Model 240, the Coleman Hitachi 124, or the Cary Model 14 spectrophotometers. Titrations were carried out at the pH indicated so as to avoid precipitation of the protein.

Fluorescence spectra were measured with instrumentation assembled in these laboratories (D'Anna and Tollin, 1971).

Circular dichroism (CD) spectra were recorded using a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment.

Amino acid analyses were performed on a Beckman Model 120C analyzer after 20-hr hydrolysis in 6 N HCl at 105°. Tryptophan and cysteine were not determined.

The sulfhydryl group was blocked (and assayed) by the method of Ellman (1959). When blocking of the sulfhydryl group was to be followed by *N*-bromosuccinimide oxidation, the sample was dialyzed against 0.025 M pH 7 phosphate buffer to remove excess Nbs_2 and the pH adjusted to the desired value before addition of *N*-bromosuccinimide.

Disc gel electrophoresis runs were performed with the Conalco Model 12 system. A 7% acrylamide gel (stacking at pH 8.9, separating at pH 9.5) was used. Samples were run for 1 hr at 100–150 V and stained with Amido Black.

FMN binding capacity was measured by diluting 10 μl of the treated holoprotein to 2 ml in 0.025 M pH 7 phosphate buffer and determining the fluorescence of released FMN (relative to a standard FMN solution equivalent to 100% release). For FMN binding to modified apoprotein, 10-μl aliquots were diluted to 2 ml in an FMN solution (in 0.025 M pH 7 phosphate buffer) to give a 1:1 ratio of protein to FMN and the residual fluorescence was measured. Excitation was at 360 nm, with emission measured at 525 nm.

Results and Discussion

Tryptophan Oxidation by N-Bromosuccinimide. Of the four tryptophans in *Azotobacter* flavodoxin (Edmondson and Tollin, 1971a), two to three residues can be oxidized by *N*-bromosuccinimide in the native holoprotein in phosphate buffer and the apoprotein in Tris buffer, as determined from the change in optical density at 280 nm (Figure 1). The holoprotein in Tris buffer was found to be much more resistant to *N*-bromosuccinimide oxidation; only 0.5–0.6 tryptophan could be titrated in this buffer. Although the general shapes of the *N*-bromosuccinimide titration curves in Figure 1 are similar for both apo- and holoproteins, at least some tryptophans are clearly more accessible in the apoprotein. This is shown by the observation that the reaction proceeds further with less consumption of *N*-bromosuccinimide with the apoprotein than with the holoprotein. Furthermore, less time is required for each aliquot of *N*-bromosuccinimide to complete its increment of oxidation of the apoprotein (1–2 min) than of the holoprotein (4 or more min).

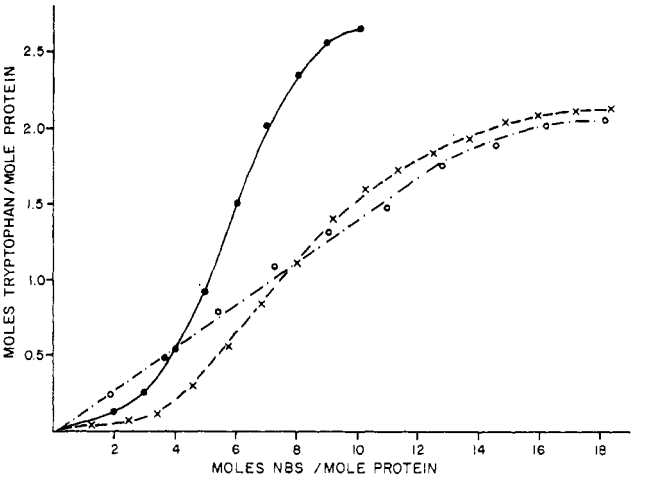

FIGURE 1: The oxidation of tryptophan residues by *N*-bromosuccinimide (NBS): (●) *Azotobacter* apoflavodoxin; (×) *Azotobacter* flavodoxin; (○) trypsin. Protein concentrations were 1 mg/ml; 0.025 M pH 6.8 phosphate buffer was used for flavodoxin, 0.1 M pH 8.5 Tris buffer adjusted to pH 6.8 with glacial acetic acid was used for apoflavodoxin and 0.1 M pH 4 acetate buffer was used for trypsin. Proteins were titrated with 10-μl aliquots of 10^{-2} M *N*-bromosuccinimide in water.

The leveling off (or occasionally slight decrease) noted in the number of tryptophans oxidized at the maximum may be due to an increase in the absorbance at 280 nm resulting from bromination of the oxindole or of tyrosine (Spande and Witkop, 1967), or to the gradual development of turbidity. Either of these processes might prevent the observation of further oxidation of tryptophan. Such appears to be the case, at least with the holoprotein. Thus, extrapolation of the data for a typical experiment, in which the maximum observed corresponded to the oxidation of 2.33 tryptophans, leads to 100% inactivation at 3.1 tryptophans modified (see Figure 4 below).

The lag phase seen in Figure 1 appears to be due to preferential oxidation of the single sulfhydryl group in the protein (Edmondson and Tollin, 1971a) by *N*-bromosuccinimide. Trypsin, in which the sulfhydryls all form intermolecular disulfides (Kauffman, 1965), does not show the lag phase.[2] In addition, the lag phase is essentially eliminated by prior reaction of the protein with dithiobis(nitrobenzoic acid), a reagent

[1] Abbreviation used is: Nbs_2, dithiobis(nitrobenzoic acid).

[2] Only 2.3 tryptophans were oxidizable in trypsin in these experiments, as compared to 3.6 reported by Spande *et al.* (1966). This may be due to differences in experimental conditions or procedures.

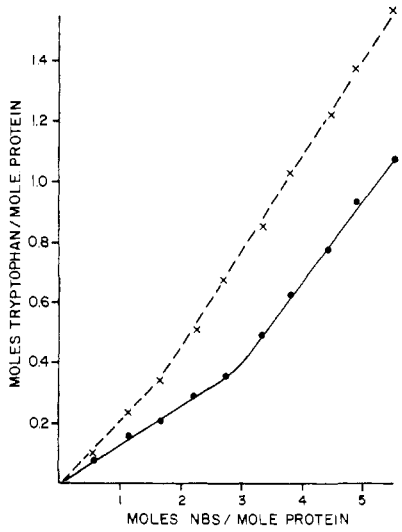

FIGURE 2: The effect of dithiobis(nitrobenzoic acid) on the oxidation of tryptophan residues in *Azotobacter* flavodoxin by *N*-bromosuccinimide: (●) apoprotein; (×) apoprotein and Nbs_2. Apoprotein was 1 mg/ml in 0.025 M pH 7.0 phosphate buffer. Titration was with 0.5×10^{-2} M *N*-bromosuccinimide in water.

which blocks free sulfhydryl groups (Ellman, 1959). This is shown in Figure 2.

FMN Binding by Oxidized Protein. The results of FMN fluorescence measurements in the presence of modified proteins indicate that tryptophan oxidation leads to a loss in the ability of the apoprotein to bind FMN (Figure 3) and to the release of bound FMN from the holoprotein (Figure 4). Essentially no FMN binds to the apoprotein (100% inactivation) when two tryptophans are oxidized by *N*-bromosuccinimide. With holoprotein, 60–70% of the FMN is released when two tryptophans are oxidized. These data can be analyzed by the technique of Tsou (1962) as reviewed by Paterson and Knowles (1972). The simplest case assumes that, if all reacting residues are oxidized at the same rate, $a = x^i$, where a is the fraction of activity (*i.e.*, flavine binding) remaining, x is the fraction of modifiable residues unchanged, and i is the number of residues essential to activity. Alternatively, a can be defined as the fractional inactivation and x as the fraction of modifiable residues which has been changed.

Figure 3 shows the results expressed as % inactivation of apoprotein *vs.* the number of tryptophans oxidized, superimposed on theoretical curves for $i = 1$, 2, and 3 for a situation in which 2.2 tryptophans are modifiable. Figure 4 shows the

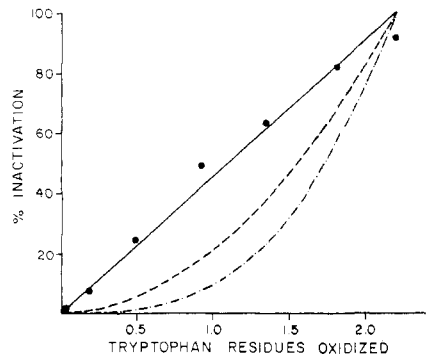

FIGURE 3: Theoretical inactivation curves based on $a = x^i$ for a total of 2.2 tryptophans modifiable in apoprotein: (——) $i = 1$; (- - - -) $i = 2$; (—·—) $i = 3$; (●) experimental points.

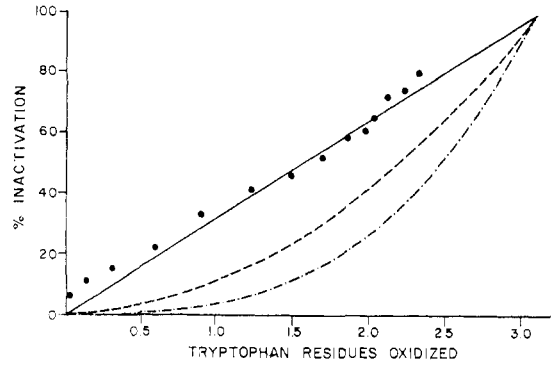

FIGURE 4: Theoretical inactivation curves based on $a = x^i$ for a total of 3.1 tryptophans modifiable in holoprotein: (—) $i = 1$; (- - -) $i = 2$; (—·—) $i = 3$; (●) experimental points.

data for holoprotein along with theoretical curves for a situation in which 3.1 tryptophans are modifiable. In both instances, the experimental points lie along the curve where $i = 1$, indicating that modification of a single tryptophan leads to a loss of FMN binding ability.

Circular Dichroism Spectra. *N*-Bromosuccinimide treatment corresponding to an oxidation of 1.3 tryptophans in holo- or apoprotein resulted in the elimination of the positive CD bands at 282, 288, and 279 nm and the negative band at 265 nm (Figure 5). In the far-uv region (Figure 5), the negative band at 222 nm increased somewhat in intensity and the negative band at 208 nm increased even more and broadened. The positive band at 195 nm decreased in intensity and shifted toward 190 nm. These results can be interpreted as indicating a conformational change upon tryptophan oxidation (perhaps an increase in α-helix content and a decrease in the amount of β structure); however, the effect of oxindole absorption on the far-uv CD spectra cannot be adequately assessed (Strickland *et al.*, 1973). Although a titration of the CD changes was not performed, these results suggest that one to two tryptophyl residues provide the major contribution to the near-uv dichroism of the holo- and apoproteins.

Disc Gel Electrophoresis of Modified Proteins. Disc gel electrophoresis runs showed that apoprotein and holoprotein were each converted to a single new species upon oxidation by *N*-bromosuccinimide. Both modified proteins migrated faster toward the anode than either the apoprotein or holoprotein. The oxidized holoprotein migrated with the fastest rate.

Amino acid analysis was used to check for modifications of residues other than tryptophan. Cysteine and tryptophan were not included in the analyses because of destruction during hydrolysis. However, a peak corresponding to about one-half residue of cysteic acid was found in samples which had been treated with *N*-bromosuccinimide, indicating modification of cysteine. This supports the results obtained with Nbs_2 (see below). The only other residue which appeared to be modified by *N*-bromosuccinimide treatment was tyrosine in the apoprotein. In this case, a change in the number of tyrosine residues found from 5 to 3 occurred. It is not known if this had a significant effect on FMN binding.

Protein Fluorescence. Apoprotein, when excited at 280 nm, fluoresces with a maximum at about 350 nm (D'Anna and Tollin, 1971). Addition of excess FMN quenches this fluorescence by about 90%, which is consistent with the fluorescence properties of the native holoprotein (D'Anna and Tollin, 1971). The tryptophan fluorescence emission intensity of *N*-bromosuccinimide-treated apoprotein or of apoprotein

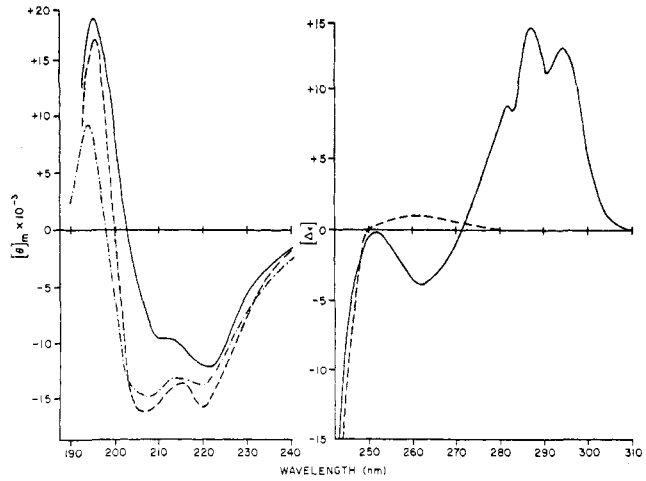

FIGURE 5: Circular dichroism spectra of modified (1.3 tryptophans oxidized) and unmodified apoflavodoxin in the far- (left) and near- (right) ultraviolet regions: (—) unmodified apoprotein; (– – –) modified apoprotein; (—·—) apoprotein from modified holoprotein. Curves for modified apoprotein and apoprotein from modified holoprotein coincide in the near-uv region. A mean residue weight of 130 was used to calculate the mean residue ellipticity in the far-uv region. Samples were in 0.025 M pH 7.0 phosphate buffer.

from *N*-bromosuccinimide-treated holoprotein was also decreased by 80–90%. No changes in the emission spectra were noted after oxidation. Using the technique of Tsou described above, we found that modification of a single tryptophan residue can account for essentially all of the protein fluorescence decrease (Figure 6). Previous work (D'Anna and Tollin, 1971) has shown that the "tryptophan quantum yield" (Teale and Weber, 1957) of the *Azotobacter* apoflavodoxin is approximately 1.5 times that of free tryptophan in aqueous solution. Thus, the tryptophyl residue which is responsible for most of this emission must be in an environment which significantly enhances its fluorescence yield.

These results can be interpreted in terms of the above-mentioned determination that one tryptophan residue is essential to the binding of FMN, if one assumes that this same tryptophan residue is present in the coenzyme binding site and is highly fluorescent in the apoprotein. Alternatively, two distinct tryptophans could be involved in these separate experiments. If this is the case, then one of these residues must be involved in binding FMN but nonfluorescent in either the apoprotein or the holoprotein. The other tryptophan must be fluorescent and be quenched by the binding of FMN, but not directly involved in the binding process.

Sulfhydryl Group Modification. The presence of one free sulfhydryl group in the holoprotein (Edmondson and Tollin, 1971a) was confirmed by titration with Nbs_2. Like tryptophan, the sulfhydryl group appeared to be much more accessible in the apoprotein than in the holoprotein on the basis of the amount of Nbs_2 required in the titration (Figure 7). No free sulfhydryl was evident after the protein was treated with *N*-bromosuccinimide. This is consistent with our observation that the lag phase obtained upon *N*-bromosuccinimide oxidation was eliminated upon treatment with Nbs_2 (Figure 2).

Titration of the sulfhydryl group with Nbs_2 had no effect on the rebinding of FMN by apoprotein or on the binding of FMN by the holoprotein. This is in agreement with the results of Hinkson (1968). Edmondson (unpublished work) in our laboratory has also observed similar behavior upon iodoacetamide treatment to modify the sulfhydryl group.

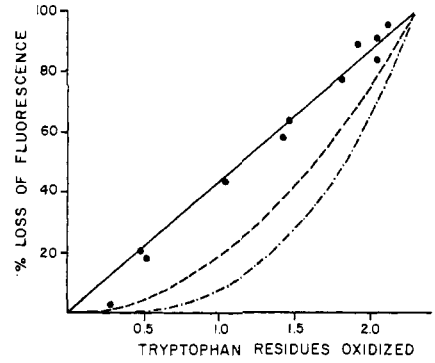

FIGURE 6: Theoretical curves of loss of fluorescence based on $a = x^i$ for a total of 2.3 tryptophans oxidized in apoprotein: (—) $i = 1$; (– – –) $i = 2$; (—·—) $i = 3$; (●) experimental points.

Holoprotein may be reduced to the semiquinone stage with excess sodium dithionite (Edmondson and Tollin, 1971b). Several minutes are normally required for this. However, when the holoprotein is first blocked by Nbs_2 the reduction to form the semiquinone is almost instantaneous. These results indicate that modification of the sulfhydryl group does affect the redox properties of the holoprotein. The CD spectrum of the modified holoprotein is virtually identical to that of the native holoprotein indicating no major changes in either flavine environment or protein conformation. It is possible that the bulky thiobisnitrobenzoic acid group could cause small changes in protein structure which are sufficient to allow more rapid chemical reduction. However, the reoxidation of the semiquinone caused by bubbling air through the protein solution appears to occur at a similar rate for either the blocked or unblocked protein. Further work is required in order to clarify the role of the sulfhydryl group in FMN redox behavior.

Conclusions

The results of the present study have provided a clear demonstration that the oxidation of a single tryptophan residue in *Azotobacter* flavodoxin leads to a loss in the ability of the protein to bind FMN. This could be either a direct effect resulting from an alteration in a critical flavine–tryptophan interaction in the binding site, or an indirect effect mediated by a conformation change in the protein. We have also shown that most (~90%) of the fluorescence of the apoprotein is due to a single tryptophan residue. This correlates well with the tryptophan fluorescence quenching observed upon FMN

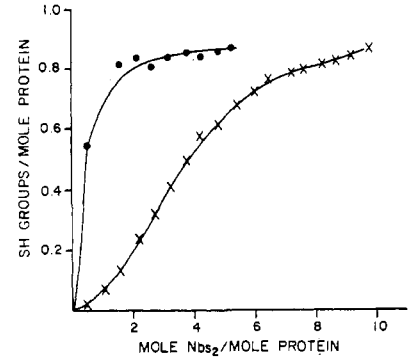

FIGURE 7: Sulfhydryl groups found by dithiobis(nitrobenzoic acid) titration: (●) apoprotein; (×) holoprotein. Apoprotein was 0.5 mg/ml in 0.1 M pH 8.5 Tris buffer. Holoprotein was 0.5 mg/ml in 0.05 M Tris–0.012 M phosphate buffer (pH 8.2). Titration was with 10-μl aliquots of 2×10^{-3} M Nbs_2 in 0.025 M pH 7.0 phosphate buffer.

binding and thus provides some support for the conclusion that a tryptophan side chain is present in the flavine binding site of the protein. Additional information regarding these observations comes from protein fluorescence lifetime measurements (Andrews *et al.*, manuscript in preparation) which demonstrate that the quenching of tryptophan fluorescence upon FMN binding to the apoprotein cannot be explained in terms of energy transfer and thus occurs at the ground state level. This could result from an interaction between isoalloxazine and indole rings, although a protein conformation change could be involved here as well. The X-ray crystallographic results obtained with *D. vulgaris* flavodoxin (Watenpaugh *et al.*, 1973) have shown that the binding site tryptophan is hydrogen bonded to a serine residue which in turn is hydrogen bonded to the ribityl phosphate group of FMN. Thus, modification of this residue would be expected to disrupt the binding of both the isoalloxazine ring and the side chain of the cofactor. It is also significant that one side of the indole ring is easily accessible to the solvent in the holoprotein, and thus would be expected to be reactive toward *N*-bromosuccinimide. Finally, it is important to point out that the *Azotobacter* and *D. vulgaris* flavodoxins belong to the same subclass of this group of flavoenzymes (D'Anna and Tollin, 1972). One of the criteria for this assignment is the nature of the visible circular dichroism spectra, which is a direct reflection of the flavine environment in the protein binding site. In conclusion, although it is clearly possible that a tryptophan side chain is present in the FMN binding site of *Azotobacter* flavodoxin, further work will be required in order to unambiguously establish this.

References

D'Anna, J. A., Jr., and Tollin, G. (1971), *Biochemistry 10*, 57.

D'Anna, J. A., Jr., and Tollin, G. (1972), *Biochemistry 11*, 1073.

Edmondson, D. E., and Tollin, G. (1971a), *Biochemistry 10*, 124.

Edmondson, D. E., and Tollin, G. (1971b), *Biochemistry 10*, 133.

Ellman, G. (1959), *Arch. Biochem. Biophys. 82*, 70.

Hinkson, J. W. (1968), *Biochemistry 7*, 2666.

Hinkson, J. W., and Bulen, W. A. (1967), *J. Biol. Chem. 242*, 3345.

Kauffman, D. L. (1965), *J. Mol. Biol. 12*, 929.

Mayhew, S. G. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press, p 185.

McCormick, D. B. (1970), *Experientia 26*, 243.

Paterson, A. K., and Knowles, J. R. (1972), *Eur. J. Biochem. 31*, 510.

Spande, T. F., Green, N. M., and Witkop, B. (1966), *Biochemistry 5*, 1926.

Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.

Strickland, E. H., Wilchek, M., and Billups, C. (1973), *Biochim. Biophys. Acta 303*, 28.

Teale, F. J. W., and Weber, G. (1957), *Biochem. J. 65*, 476.

Tsou, C. L. (1962), *Sci. Sin. 11*, 1535.

Watenpaugh, K. D., Sieker, L. C., and Jensen, L. H. (1973), *Proc. Nat. Acad. Sci. U. S.* (in press).