First publ. in: Flavins and flavoproteins : International Symposium on Flavins and Flavoproteins, Brighton, England, July 9 - 13, 1984, pp. 769-772

4-THIOFLAVINS AS ACTIVE SITE PROBES OF FLAVOPROTEINS: REACTION WITH SULFITE AND FORMATION OF 4-HYDROXY-4-SULFONYLFLAVINS

Al Claiborne, Vincent Massey
Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109, USA

Monika Biemann, and Sandro Ghisla
akultät für Biologie der Universität Konstanz, D 7750 Konstanz

Introduction

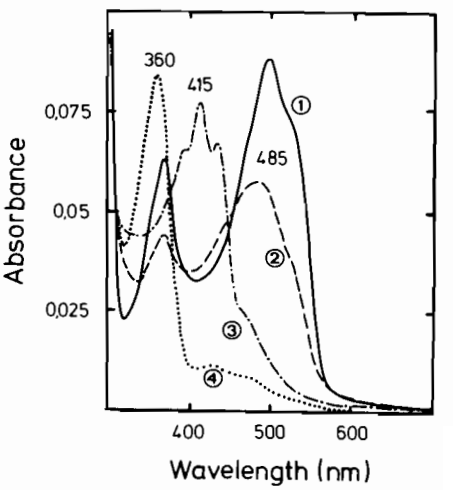
4-Thioflavincoenzymes are useful as active site probes for a variety of flavoproteins (1). This results from their high reactivity at C(4)=S with either nucleophiles or electrophiles, which has allowed investigation of the interaction of the flavin N(3)-C(4)=O regions with the apoprotein and its accessibility from the solvent. Significantly different reactions with sulfite have been observed with different flavoenzyme classes reconstituted with 4-Thio-FAD or -FMN. 4-Thio-FAD-D-amino acid oxidase and 4-thio-FMN-lactate oxidase react rapidly to yield "classical" N(5)sulfite adducts (Fig. 1, Curve 4) as do the native enzymes (2). 4-Thio-FAD-p-hydroxy-benzoate hydroxylase on the other hand, reacts slowly and biphasically to yield a product (Fig. 1, Curve 3) the spectral properties of which are typical of 3,4-dihydroflavins (5). In view of possible relevance for classifying different types of flavoenzymes, we studied the reaction of free 4-thioflavins with sulfite and elucidated the structure of the products as 4-hydroxy-4-sulfonyl flavins.

Results and discussion

4-Thio-FAD-D-amino acid oxidase reacts with sulfite rapidly and reversibly to yield a fluorescent species with an absorption maximum at 360 nm (Fig. 1, Curve 4). This species can reasonably be ascribed to a flavin-N(5)-sulfite adduct, which in the case of native enzyme has been shown to exist in a reversible equilibrium (3). The reaction of 4-thio-FAD-p-hydroxy-benzoate hydroxylase with sulfite on the other hand is slow and biphasic. A first intermediate is formed in a sulfite concentration dependent reaction (Fig. 1, Curve 2), which is converted to a final blue fluorescent species with an absorption maximum at 415 nm (Fig. 1, Curve 3).

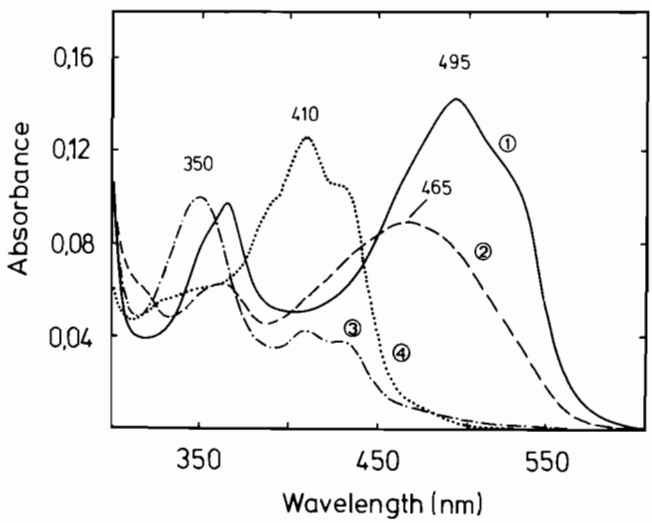
Figure 1: Effect of sulfite on 4-thio-FAD reconsituted enzymes

Curve 1; 4-thio-FAD-p-hydroxy-benzoate hydroxylase in 0.05 M phosphate, pH 7, 25°C. Curve 2; 2.5 min after addition of 10 mM sodium sulfite. Curve 3; after incubation for 20 hr at 25°C. Curve 4; N(5)-sulfite adduct of 4-thio-FAD-D-amino acid oxidase in 0.1 M phosphate, pH 7.0, 4°C.



When 4-thioriboflavin is mixed aerobically with sodium sulfite, a blue fluorescent species with absorption maximum at 410 nm (Fig. 2. Curve 3) is formed. The reaction is biphasic and sulfite concentration dependent, and proceeds via formation of a transient species with absorption maximum at 465 nm (Fig. 2, Curve 2). Under anaerobic conditions the same intermediate is formed, but the final product is a typical N(5)-sulfite flavinadduct (Fig. 2, Curve 3). Addition of riboflavin binding protein to the first intermediate restores 84 % of the starting material, demonstrating that the

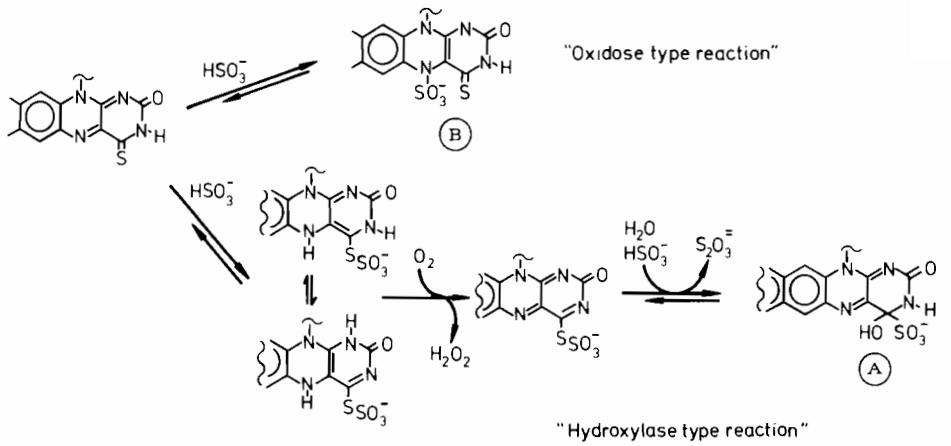
C(4) sulfur is not lost at this stage of reaction. In the final product, however no sulfur at the S^{-2} oxidation level is present at C(4). N(3) blocked and unblocked 4-thioflavins react similarly. This excludes a flavin-4-sulfonyl structure for the sulfite adducts. Their chromatographic properties indicate the presence of a negative charge on the flavin. The flavin sulfite adducts are extremely photolabile, they yield normal oxidised flavin and sulfite. They are stable if kept in the dark. Anaerobic or aerobic photolysis leads quantitatively to normal oxidized flavins and not to the reduced one as it should be expected from a 3,4-dihydroflavin.



igure 2: Effect of sulfite on 4-thioriboflavin under aerobic and anaerobic conditions.
Curve 1; 4-thioriboflavin in oxygen free 0.1 M phosphate, pH 7.0, 0 - 1°C in an anaerobic cuvette at the same concentrations as the remaining curves. Curve 2; 80 - 160 sec after addition of 0.18 M sodium sulfite. Curve 3; 12 hr later. Curve 4; 4-thioriboflavin in aerobic phosphate, pH 7.0, 12 hr after addition of 0.18 M sodium sulfite.

In the presence of 5 % H_2O_2, in 1 N acetic acid, or with meta-chlorperbenzoate or methylmethanthiolsulfonate at pH 7, the sulfite adducts are stable. When irradiated in the presence of amines they undergoe nucleophilic substitutions to yield the corresponding 4-amino- or 4-imino-flavins similar to those observed with 4-thioflavins (1,4). While the sulfite adducts have similar UV-VIS and fluorescence properties as 3,4-di-

hydroflavins (5), unlike them they show no ^{1}H-NMR resonance which could be attributed to a C(4)-H function. These properties indicate that the flavin sulfiteadducts have an sp^3 center at the C(4) position, and deduce that they have a 4-hydroxy-4-sulfonyl structure. 4-Thio-FAD reconsituted p-hydroxybenzoate hydroxylase forms 4-hydroxy-4-sulfonylflavins with sulfite like the free coenzyme under aerobic conditions (Scheme below, "hydroxylase" reaction), while 4-thio-FAD / -FMN reconstituted oxidases or 4-thioflavins under anaerobic conditions form N(5)-sulfite adducts (Scheme below, "oxidase" reaction).



References

1. Massey, V., Claiborne, A., Biemann, M., and Ghisla, S.: J. Biol. Chem in press.
2. Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., and Foust, G. P.: J. Biol. Chem. 244, 3999 - 4006 (1969).
3. Müller, F., and Massey, V.: J. Biol. Chem. 244, 4007 - 4016 (1969)
4. Müller, F., and Hemmerich, P.: Helv. Chim. Acta 49, 2352 - 2364
5. Müller, F., Massey, V., Heizmann, C., Hemmerich, P., Lhoste, J-M., and Gould, D. C.: Eur. J. Biochem. 9, 392 - 401 (1969)
6. Hemmerich, P., and Erlenmeyer, H.: Helv. Chim. Acta 40, 181 - 186 (1957)