Oxidation-Reduction Properties of the 8α-Substituted Flavins*

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Dale E. Edmondson† and Thomas P. Singer
From the Department of Biochemistry and Biophysics, University of California, San Francisco, California, 94143, and Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121

SUMMARY

The oxidation-reduction potentials of a variety of riboflavin derivatives substituted at the 8α position have been determined by anaerobic titration with dithionite in the presence of suitable dyes and spectrophotometric determination of the ratio of oxidized to reduced flavin. The potentials of 8α-substituted flavins, including the histidyl-8α-riboflavin component of succinate dehydrogenase and the cysteiny1-8α-riboflavin component of monoamine oxidase, were found to be 0.02 to 0.03 volt higher than the potential of riboflavin. In accord with this, the affinity of the 8α-substituted flavins for sulfite was 10 to 20 times higher than that of riboflavin. This process follows first order kinetics with a pH optimum of 6.0 at 25°C.

Reduction by dithionite to the flavin hydroquinone proceeded with a 2 electron uptake and was completely reversible on admission of O2 in each case, except for the 8α-8-cysteinyl-sulfone of riboflavin. On reduction of the latter by equimolar dithionite, cysteinesulfinate was spontaneously eliminated and riboflavin was formed. This process follows first order kinetics with a pH optimum of 6.0 at 25°C.

During the past few years the chemical nature of the linkage of covalently bound flavin to the protein components of several enzymes has been elucidated. In each case studied, the peptide chain is attached at the 8α position of riboflavin. In succinate dehydrogenase (1-3) and in D-hydroxyisocitrate oxidase (4), attachment of the flavin is to the N(3) position of the imidazole ring of histidine. The flavin site of liver monoamine oxidase contains 8α-cysteiny1 FAD, and the cysteine is in a thioether linkage to the 8α-CH2 group (5, 6). Chromatium cytochrome c52 contains 8α-c-cysteinyl FAD, and the cysteine is in a thioether linkage to the N(3) position of the imidazole ring of histidine. The flavin site of liver monoamine oxidase contains 8α-cysteinyl FAD, and the cysteine is in a thioether linkage to the 8α-CH2 group (5, 6). Chromatium cytochrome c52 contains 8α-c-cysteinyl FAD, and the cysteine is in a thioether linkage to the N(3) position of the imidazole ring of histidine. The flavin site of liver monoamine oxidase contains 8α-cysteinyl FAD, and the cysteine is in a thioether linkage to the 8α-CH2 group (5, 6).

Although in the course of these studies a considerable amount of information has accrued on the chemical reactivity, fluorescence characteristics, absorption and electron spin resonance spectra, photochemistry, and chromatographic properties of 8α-substituted flavins, their oxidation-reduction potentials have not been measured. Knowledge of the oxidation-reduction potentials of naturally occurring 8α-substituted flavins was of interest, since it seemed conceivable that covalent linkage to the protein increases the potential of the flavin and thereby facilitates catalysis. A case in point is succinate dehydrogenase, where the Em,0 of the substrate pair is some 0.250 volt higher (15) than that of the FAD-FADH2 couple. This paper deals with the electron affinity of the isoxalazine ring system in various 8α-substituted flavins, as measured by oxidation-reduction potentials and sulfite affinities (16).

In addition, we describe a reductive 8α elimination reaction, which may be of importance in the catabolism of covalently linked flavins.

EXPERIMENTAL PROCEDURE

Materials—Flavins containing various 8α substituents were synthesized utilizing 8α-BrTARF (3) as described by Kenney and Walker (14) and by Glusia and Hemmerich (13). The formylriboflavin compound was a gift from Dr. G. Blankenhorn (The University of California, Davis).

8α-S-Cysteiny1sulfone-TARF was synthesized by incubating 10 mg of cysteinesulfonic acid (65 m) with 20 mg of 8α-BrTARF (40 μ) in 0.1 ml of dimethylformamide for 7 days at room temperature under anaerobic conditions. The product was then isolated by preparative high voltage electrophoresis at pH 1.6 with a final yield of 33%. The flavin sulfone prepared in this manner was identical in spectral properties with the material prepared by treating 8α-cysteinylriboflavin with oxidizing agents (3, 13).

Purity of the various substituted flavins were monitored by high voltage electrophoresis, by thin layer chromatography, and by descending paper chromatography under conditions previously described (2, 3, 14). The 8α-sulfur-containing flavins were unstable over prolonged storage in solution, and thus experiments were performed immediately after purification. The flavins thus synthesized and purified were identical in spectral properties (absorption and fluorescence) as well as electrophoretic mobility with those previously reported (12-14).

Anaerobic Titration—All anaerobic experiments were performed in an atmosphere of helium that had been purified over hot copper and water-saturated in a gas train made of glass and...
butyl rubber tubing. Dithionite solutions were prepared anaerobically in a glass vessel and stored in a gas-tight Hamilton syringe. Anaerobic titrations were performed in a glass cuvette similar to that reported by Burleigh et al. (17) but modified in that no serum stoppers were used. Dithionite solutions were standardized by anaerobic titration of a solution of 3-methylumbelliferyl flavin (a gift of Dr. S. Ghisla, The University of Michigan). Flavin reduction was monitored by measuring visible absorption spectra with a Cary 14 spectrophotometer thermostatted at 25°C after the addition of each aliquot of dithionite.

The oxidation-reduction potentials of the various 8α-substituted flavins were measured by anaerobic dithionite titration of a mixture of the appropriate flavin and of indigo disulphonate ($E_{m,7} = -0.116$ volt) or, in some cases, of anthraquinone-2,6-disulphonate ($E_{m,7} = -0.184$ volt) (15). The indigo dye is advantageous in that its reduction is easily monitored at 610 nm where there is no flavin absorption and has an isosbestic point at 460 nm, near the absorption maximum of oxidized flavin. The spectrum of the reduced anthraquinone dye overlaps considerably with those of oxidized and reduced flavins. Dye reduction could be monitored by the decrease in absorbance at the flavin isosbestic point (around 330 nm), and flavin reduction could be monitored at the dye isosbestic of 352 nm. The oxidation-reduction potential for the system at equilibrium at various stages of reduction was calculated using the equation:

$$E_h = E_{m,7} - \frac{RT}{N\pi} \ln \left( \frac{[\text{oxidized dye}]}{[\text{reduced dye}]} \right)$$

The amounts of reduced and oxidized flavin were determined as described and the oxidation-reduction potential ($E_{m,7}$) was determined from a plot of $E_h$ versus log [oxidized flavin]/[oxidized flavin].

### Determination of Sulfite Affinities

The binding of sulfite to the various 8α-substituted flavins was determined as described by Müller and Massey (16) in 0.3 M P$_i$, pH 7.0, at 25°C. The absorption coefficients of the flavin-sulfite complexes at 450 nm were estimated from Benesi-Hildebrand plots (18) and were in the range of 500 to 1000 M$^{-1}$ cm$^{-1}$.

Reversibility of the reaction was tested by acidification (pH ~0) of a solution of flavin plus excess sulfite, bubbling with helium for 1 hour to remove the resulting SO$_2$, neutralization, and measurement of the spectrum of the resulting solution. In all cases the resulting absorption spectrum (after correcting for dilution) was identical with that of the untreated flavin.

### RESULTS

#### Stoichiometry of Electron Uptake

To ascertain the stability of the 8α-substituted flavins to reduction, dithionite titrations were performed. The presence of an isosbestic point during reduction and subsequent reoxidation by O$_2$ with return of the original absorption spectrum, establishes the oxidation-reduction reversibility of the flavins. 8α-Histidylriboflavin is reduced by 1 M equivalent of dithionite to its hydroquinone form, with an isosbestic point at 327 nm (Fig. 1). Subsequent reoxidation by air gave an absorption spectrum identical with that of unreduced flavin. This behavior was observed with all of the 8α-substituted flavins tested (Table I), with the exception of 8α-S-cysteinylsulfone-TARF, which was unstable to reduction (see below).

#### Interaction of Sulfite with 8α-Substituted Flavins

Müller and Massey (16) have shown that flavins form adducts with sulfite at the N(5) position, which may be considered a sulfamic acid of 1,5-dihydroflavin (19). The absorption spectral properties of flavin-sulfite adducts are similar to, but not identical with, those of flavin hydroquinone. It has also been demonstrated (16) that the oxidation-reduction potentials of various flavins correlate well with their respective sulfite affinities. The degree of sulfite affinity for the various 8α-substituted flavins is, therefore, an indication of their oxidation-reduction potentials.

Fig. 2 illustrates the absorption spectra of 8α-histidylriboflavin in the visible range, in the presence of various concentrations of sulfite. In a manner analogous to unsubstituted flavins (16), the 450 nm band is bleached with increasing sulfite concentration and an isosbestic point is observed, denoting the presence of only two components in solution (oxidized flavin and the sulfite adduct). Comparison with the data of Müller and Massey...
Fig. 2. Spectral changes at equilibrium upon adding sodium sulfite to 8a-N-3-histidylriboflavin in 0.3 M Pi, pH 7.0, at 25°. Curve 1, no addition of sulfite; Curve 2, 0.02 M SO₃⁻⁺ + HSOS⁻⁻; Curve 3, 0.1 M SO₃⁻⁺ + HSOS⁻⁻; Curve 4, 0.4 M SO₃⁻⁺ + HSOS⁻⁻; Curve 5, 1.0 M SO₃⁻⁺ + HSOS⁻⁻. The reference cell contained a concentration of sulfite equal to that of the sample cuvette.

Flavin-sulfite complex formation followed a pseudo-first order rate, which was dependent on sulfite concentration. As may be seen in Table I, with one exception, substitution in the 8a position brings about a 2- to 3.0-fold increase in the reaction rate of flavins with sulfite, but the values for the dissociation constant decreased 10- to 20-fold. Substitution at the 8a position thus makes the isoalloxazine ring more electron deficient and the decrease in KD values is mainly due to a decrease in koff rather than an increase in the “on” kinetic rate constant. Of particular interest is that the nature of the &Y substituent has little influence on the sulfite affinity of the N(5) position.

Oxidation-Reduction Potential Measurements—From the data of Müller and Macrow (16) the sulfite affinities of the 8a-substituted flavins would suggest oxidation-reduction potentials in the area of ~0.16 volt. Anaerobic dithionite titrations of a mixture of indigo disulfonate or anthraquinone-2,6-disulfonate with the various 8a-substituted flavins gave E_m, values ranging from ~0.153 volt for 8-formylriboflavin to ~0.170 volt for 8-hydroxyriboflavin (Table I), in agreement with the values suggested by the dissociation constants of the sulfite complexes. The plots in Fig. 3 show the linear relation between potential and the logarithm of the ratio of oxidized to reduced flavin with slopes as expected for a 2-electron reduction. The agreement of the individual points with the theoretical line show the reliability of the oxidation-reduction measurements using this experimental method. In accord with the sulfite data, the nature of the 8a substituent had little effect on the midpoint potentials, although they were 0.03 to 0.04 volt higher than for the corresponding unsubstituted flavins.

Reductive Cleavage of 8a-S-Cysteinylsulfone-riboflavin—Dithionite reduction of 8a-S-cysteinylsulfone-riboflavin or its tetra-acetyl form and subsequent reoxidation by air gave rise to a flavin compound with a near ultraviolet absorption maximum at 372 nm, as compared with a maximum at 352 nm in the untreated material. A similar behavior is observed upon reduction of this flavin by light EDTA. High voltage electrophoresis of the reduced flavin (by either dithionite or light EDTA) showed no migration at pH values of 1.6, 3.5, and 6.2, conditions under which the untreated material does migrate. The untreated flavin was ninhydrin-positive, while the flavin, treated as above, became ninhydrin-negative, showing the loss of the &N₃ group. A ninhydrin-positive spot was observed, however, which migrated differently from the flavin, with RF value identical with that of cysteic acid. Formation of cysteic acid in this experiment was probably due to the oxidation of cysteine sulfinic acid to form the reduced flavin by oxygen. Thin layer chromatography of the modified flavin identified it as riboflavin.

To investigate the mechanism of elimination of the 8a substituent, a dithionite titration was performed on 8a-S-cysteinylsulfone-TARF. Upon the addition of the first aliquot of dithionite, 0.15 mole per mole of flavin, the 450 maxima was immediately bleached partially. Subsequently, however, it slowly increased in intensity to a value identical with that observed before the addition of dithionite. The absorption spectra showed a shift in the near ultraviolet band to the red (Fig. 4A). Subsequent additions of dithionite up to 2 moles per mole of flavin exhibited the same behavior with shift of the near ultraviolet band to 372 nm (Fig. 4A). The isosbestic point at 363 nm indicates the presence of two species of oxidized flavin in solution. Subsequent additions of dithionite up to 2 moles per mole of flavin bleached the flavin spectrum with an isosbestic point at 330 nm (Fig. 4B). The titration plot in Fig. 5 shows that 2 electrons are required for reductive cleavage of the 8a substituent and 2 electrons for the reduction of the resulting flavin compound.

To provide additional evidence that this observation is indeed a unimolecular cleavage reaction, the kinetics was measured as a function of pH. To an anaerobic solution of 8a-S-cysteinylsulfone-TARF, 1 M equivalent of dithionite was added and the rate of increase in absorbance at 450 nm was measured. The reaction followed a first order rate through four half-lives in the

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This value for 8-formylriboflavin is for a species in which a ribityl side chain hydroxy group is linked to the 8 position via a hemiacetal linkage (D. E. Edmondson and T. P. Singer, manuscript in preparation).
FIG. 4. A, spectral changes during the anaerobic addition of the first molar equivalent of dithionite to 8a-S-cysteinylsulfone-TARF in 0.1 M Pi, pH 7.0. Upon each addition of dithionite, the spectrum was recorded after no further spectral changes were apparent. The molar equivalents of dithionite for the respective curves are: Curve 1, none; Curve 2, 0.15; Curve 3, 0.3; Curve 4, 0.6; Curve 5, 0.75; Curve 6, 1.0. The spectra are uncorrected for dilution. B, spectral changes during the anaerobic addition of a second molar equivalent of dithionite to the flavin solution in A. The total molar equivalents of dithionite added for the respective curves are: Curve 1, 1.1; Curve 2, 1.26; Curve 3, 1.43; Curve 4, 1.63; Curve 5, 2.40. The spectra are uncorrected for dilution.

pH range of 4.5 to 8.8. Increasing the flavin concentration 10-fold had no significant effect on the rate constant, as expected for a first order reaction. The rate of the reaction was strongly pH-dependent, with a maximal rate at pH 6.0 (see inset, Fig. 6), a rapid decrease at lower pH values, and a slightly slower decrease as the pH was raised. Analysis of the rate data was complex in that values on each side of the maximum were not consistent with a single proton ionization. A possible explanation for this will be elaborated upon under "Discussion."

It was of interest to see whether N(5) substitution by sulfite could also lead to the cleavage of the 8a-S-cysteinylsulfone. The sulfite titration data (Table I) indicated no irreversible changes in the flavin after removal of the bound sulfite. This experiment was repeated by incubation of the cysteinylsulfone flavin with 1.8 M sulfite for 4 hours at room temperature. Removal of the bound sulfite and spectral analysis showed properties identical with those of untreated material. Thus, N(5) substitution by sulfite does not, under these conditions, lead to cleavage of the 8a substituent.

FIG. 5. Dithionite titration of 8a-S-cysteinylsulfone-TARF in 0.1 M Pi, pH 7.0. All points are corrected for dilution. The increase in absorption at 330 nm after 2.0 M equivalents of dithionite is due to excess dithionite.

FIG. 6. First order kinetic plot of the reappearance of absorption at 450 nm after the anaerobic addition of 1 M equivalent of dithionite to 8a-S-cysteinylsulfone-TARF in 0.1 M Pi, pH 6.5, at 25°. The inset shows the dependence of the rate constant on pH. The buffers for the various pH values are: 0.1 M acetate, pH 4.5 and 5.0; 0.05 M Pi - 0.05 M acetate, pH 5.5; 0.1 M Pi, pH 6.0, 6.5, 7.0, and 7.5; 0.1 M Pi', pH 8.05 and 8.8.

The data presented in Table I show that the incorporation of a substituent in the 8a position in place of hydrogen increases the electron affinity of the isouloxazine ring in varying degrees, depending on the nature of the substituent. As shown earlier for other flavin analogs (16), the sulfite affinities of the 8a-sub-
stututed flavins correlate quite well with their respective oxidation-reduction potentials. Although there is a shift of approxi-
mately +0.03 volt in flavin oxidation-reduction potential upon
88 substitution, the nature of the substituent does not seem to
drastically influence the observed values.

The Chromatium cytochrome e550 peptic tetrapeptide (FAD
level) has a potential about 0.02 volt more positive than FA92, although the observed flavin-tyro sine interaction (9) could very
well have an influence. Indeed, the close proximity of an aro-
matic group (e.g. tyrosine or tryptophan) has been shown to
raise the flavin potential 0.05 to 0.06 volt for normal flavins (20).
The corresponding tryptic-chymotryptic flavin tripeptide in
which the COOH-terminal tyrosine is removed was not reversibly
oxidized on dithionite reduction, in agreement with an earlier
observation (8). This disparity in properties between the two
flavin peptides further points out the stabilizing influence of the
COOH-terminal tyrosine on the 8a-thiohemiacetal bond, which
is also seen on performic acid oxidation studies (9).

A development from this study which serves as a model
system for the conversion of 88-substituted flavins to “normal”
flavins is the cleavage of the 8a-cysteinylsulfone from the reduced
flavin. Previous studies have shown that histidine and cysteine
are liberated from the 88 position by strong acid hydrolysis or
by treatment with zinc in acid media (3, 5). Mild procedures,
such as flavin reduction by a stoichiometric amount of dithionite,
do not cause 88 elimination with any of the other analogs tested.

The scheme in Fig. 7 depicts a mechanism for this elimination
which is most consistent with the data. The oxidized flavin (I)
is reduced to its hydroquinone form (II). The electron rearran-
gement to the electron-deficient sulfur of the sulfone causes
elimination of the cysteinylsulfinate, leaving the flavin in a
“quinhydrone” form (III), which then tautomerizes (through IV
as a possible structure) to form the unsubstituted flavin (5).
Structure III is a flavin intermediate which has been used to
explain flavin dimerization under basic conditions (21) and to
explain the deuterium-hydrogen exchange in the 8-methyl group
when flavin is heated at neutral pH (22).

The first order nature of the reaction (Fig. 6) eliminates any
participation of oxidized flavin at later stages of the reaction.
Conceivably, oxidized flavin could be reduced to an equilibrium
concentration. However, the midpoint potentials are well
separated (see sulfite K, values, Table I), so that this possibility
does not complicate the kinetics.

The observation that sulfite addition to the N(5) position does
not lead to 88 elimination is of considerable interest, in view of its
electronic similarity with the hydroquinone form. The reason for this may lie in the geometry of the respective reduced
flavin species.

The reduced form of flavin was first postulated to be non-
coplanar, on the basis of its absorption spectra (23) and later
confirmed by x-ray crystallography (24) to be in a bent “butter-
fly” configuration, with the two planes intersecting on the N(5),
N(10) axis. To achieve maximal orbital overlap for electron
transfer to the 88-sulfone, the nitrogen at the 5 position must be
in the plane of the benzenoid ring. This may be achieved
through the all-coplanar form (a conformation which is ener-
getically unfavorable) but would not arise from other more energetically favorable mechanisms, such as ring inversion or
nitrogen inversion (25). The population of molecules in the all-
coplanar form, in accord with the Boltzmann distribution law,
would be proportional to the rate of elimination. In the case of
flavin hydroquinone, where hydrogen is the N(5) substituent,
an appreciable population may be in the all-coplanar form, while
steric considerations would suggest that the flavin-sulfite adduct
has a negligible population in this configuration.

The pH rate profile for the elimination suggests two effects
on the rate of the reaction (inset, Fig. 6). The increase in rate
as the pH is lowered from 8.8 to 6.0 may reflect the protonation
of the flavin hydroquinone. Spectral studies suggest the anionic
flavin hydroquinone is in a more bent configuration than the
neutral form (23) and thus one would expect an increase in rate
as the population of molecules in which the N(5) is planar with
the benzenoid ring increases. Below pH 6, the difficulty in re-
moving the N(5) proton increases, hence the decrease in rate.
The opposing effects of these two processes could be responsible
for the difficulty in analyzing the data in terms of a single ioniza-

The observation of an elimination from the 88 position by
simply reducing the flavin presents a possible mechanism for the
physiological conversion of 88-substituted flavins to normal
flavin, by simply modifying the substituent to make it electron-
deficient. Furthermore, this process may have implications
in the catalytic process of flavoenzymes which do not contain
covalently bound flavin. Efforts are under way to study fur-
ther these new aspects of flavin chemistry.

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