Studies of Reactions between Flavins and Quinones, Mercaptans, and Enolates

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(Z. Naturforsch. 27 b, 1016–1020 [1972]; received May 10, 1972)

Flavines, Quinones, Mercaptans, Enolates, Mechanism and Oxidation

A. Equilibrium and second-order rate constants have been measured for the redox reaction between phthaloquinone and dihydroriboflavin (and its reverse) at a number of pH values. The equilibria and kinetic determinations are in agreement. No intermediates could be found in the reaction, and it is proposed that with this and other quinones electron transfer occurs within a complex of the flavin and hydroquinone (or dihydroflavin and quinone). B. A survey of the reaction between mercaptans and flavins is reported, as well as data showing that with mercaptoethanol the reaction is reversible, the disulfide and reduced flavin giving mercaptan and flavin. C. The reaction of alkylphenone enolates with isaloalloxazine is described. This reaction leads to 50% isaloalloxazine radical anion and an adduct of the enolate with the ketone. A possible route for this process is discussed.

Unusual abbreviations: F, flavin; Rf, riboflavin; Q, quinone; 3-MeLf, 3-methylumiflavin; BPI, 3-benzyl-10-phenylisooalloxazine; H₂A, acetophenone.

A. Quinones

We have previously reported on the reactions of flavins with quinones, two typical examples of which are represented by eqns. (1) and (2).

\[
\begin{align*}
F + QH_2^+ &\rightleftharpoons F + QH_2, \\
&\text{(1)}
\end{align*}
\]

\[
\begin{align*}
F + QH_2^+ &\rightleftharpoons F + QH_2 \\
&\text{(2)}
\end{align*}
\]

These processes are extremely rapid, giving quantitative yields of products, and a mechanism involving complexing followed by electron transfer was proposed (3).

\[
Q + FH_2 \rightleftharpoons Q \cdots FH_2 \rightleftharpoons QH_2 \cdots F \rightleftharpoons QH_2 + F
\]

(3)

Since the direction of reaction (3) depended upon the oxidation potential of the flavin-quinone couple, we prepared a quinone of redox potential close to that of riboflavin (Rf) in water. Upon anaerobic mixing of either RH₂ (\(E'_0 = -0.21 \text{ v}\)) and phthaloquinone (\(E'_0 = -0.20 \text{ v}\)) or Rf and phthalhydroquinone, a pH dependent equilibrium is attained, with values as shown in Table I. The hydroxyl group of the quinone has a \(pK_a\) of 5.1 (by spectrophotometric titration), and dihydroflavin has a \(pK_a\) of 6.7. A plot of \(\log K_{eq}\) (from Table I) vs pH

<table>
<thead>
<tr>
<th>pH</th>
<th>(10^{-4} k_2)</th>
<th>(10^{-4} k_2)</th>
<th>(K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.06</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.5</td>
<td>0.20</td>
</tr>
<tr>
<td>7.93</td>
<td>0.55 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.36</td>
</tr>
<tr>
<td>7.83</td>
<td>0.55 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.54</td>
</tr>
<tr>
<td>7.21</td>
<td>6.30</td>
<td>0.54</td>
<td>6.03</td>
</tr>
<tr>
<td>6.11</td>
<td>((\sim 10)^2)</td>
<td>(\sim 10^2)</td>
<td>(\sim 10^2)</td>
</tr>
<tr>
<td>4.52</td>
<td>((\sim 10)^3)</td>
<td>(\sim 10^3)</td>
<td>(\sim 10^3)</td>
</tr>
</tbody>
</table>

All runs at 0.10 ionic strength, 0.05 M phosphate buffer. Both the flavin and quinone were about \(10^{-4} \text{ M}\) for equilibrium measurements, and varied from \(10^{-4}\) to \(10^{-5} \text{ M}\) for stopped-flow experiments. Each kinetic entry represents at least three separate experiments.
for the data above pH 7 gives an excellent linear fit with slope \(-2.0\), in agreement with eqn. (4).

\[
2\text{H}^+ + \text{Rf} + \text{F}^- + \text{RH} \rightarrow \text{Rf}^+ + \text{F} + \text{R}\text{H}^+ + \text{R}\text{H} \quad (4)
\]

Using an anaerobic stopped-flow spectrophotometer, we have also measured the rates of reaction (4) in both directions. The equations used to analyze these data are those given by LAIDLER \(^4\) for a reversible second order reaction: for \(A + B \rightarrow C + D\),

\[
k_2 \frac{2 a (a-x_0)}{x_0} t = \ln \left[ \frac{x (a-2 x_0)}{a (x_0-x)} \right] \quad (5)
\]

where \(A_0 = B_0\), \(C_0 = D_0 = 0\), and \(a = A_0\), \(x = A_0 - A_t\), \(x_0 = A_{\text{equil}}\).

The stopped-flow data gave linear fits to this equation for about 75\% of the reaction in each direction, calculated \(k_2\) values being included in Table I. The agreement between experimental \(k_2/k_{-2}\) and experimental \(K\) values is quite close, probably fortuitously so given the accuracy of the data and difficulties of the experiments.

Examination of the spectrum up to 900 nm gave no evidence for long wavelength intermediates, nor were any found during the course of the reaction on stopped-flow. As postulated in our earlier report, we favor a mechanism of complete electron transfer within a complex between the reacting moieties.

### B. Mercaptans

We have continued our study of the intermolecular oxidation of mercaptans to disulfides \(^5\) (6) in

\[
2\text{RSH} + \text{F} \rightarrow \text{RSSR} + \text{FH}_2 \quad (6)
\]

\[(R = \text{n-C}_6\text{H}_{13}, \text{C}_6\text{H}_5\text{CH}_2)\]

which complete flavin reduction and excellent yields of disulfides were obtained. These reactions are much slower than the quinone processes, are base catalyzed, and probably involve (as proposed earlier) \(^5\) nucleophilic attack by RS\(^-\) upon flavin followed by a second nucleophilic attack by another RS\(^-\) this time upon the sulfur of the initial adduct to eliminate FH\(^+\), which should be a reasonably good leaving group in such an SN\(_2\) displacement (7). At this stage we have no evidence about the site of substitution, although a recent study by BRUICE \(^6\) eliminates position 10\(a\), leaving 4\(a\) and 5 as the most likely sites.

In Table II approximate pseudo-first-order rate constants (RSH \(\gg\) F) of flavin bleaching for a variety of mercaptans in different media are presented. It is apparent that there is a wide range of reactivities among these mercaptans, and it seems that the driving force for reaction with our original thiols (n-butyl and benzyl) may have been precipitation of the disulfide as the reaction proceeded.

Indeed, with the disulfide of 2-mercaptoethanol and dihydroflavin, mercaptoethanol and flavin are obtained. The pseudo-first-order rate constants were determined at pH 10.86 (0.1 M phosphate buf-

<table>
<thead>
<tr>
<th>Flavin (^b)</th>
<th>R (^c) in RSH</th>
<th>Medium (^d)</th>
<th>pseudo (k_1) (^e) [(\text{min}^{-1} \times 10^3)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf</td>
<td>C(_6)H(_2)CH(_2)</td>
<td>I</td>
<td>5.7</td>
</tr>
<tr>
<td>Rf</td>
<td>C(_6)H(_5)</td>
<td>I</td>
<td>24</td>
</tr>
<tr>
<td>Rf</td>
<td>HO(_2)CCH(NH(_2))CH(_2)</td>
<td>I</td>
<td>0.4 (^t)</td>
</tr>
<tr>
<td>Rf</td>
<td>C(_6)H(_5)CH(_2)</td>
<td>II</td>
<td>17</td>
</tr>
<tr>
<td>Rf</td>
<td>n-C(_6)H(_5)</td>
<td>II</td>
<td>10</td>
</tr>
<tr>
<td>Rf</td>
<td>HO(_2)CCH(_2)</td>
<td>II</td>
<td>(3) (^g)</td>
</tr>
<tr>
<td>Rf</td>
<td>HO(_2)CCH(_2)</td>
<td>II</td>
<td>0.5 (^t)</td>
</tr>
<tr>
<td>Rf</td>
<td>HO(_2)CH(_2)CH(_2)</td>
<td>II</td>
<td>0.5 (^t)</td>
</tr>
<tr>
<td>Rf</td>
<td>HOCH(_2)CH(_2)</td>
<td>II</td>
<td>0.6 (^t)</td>
</tr>
<tr>
<td>3-MeLf</td>
<td>C(_6)H(_2)CH(_2)</td>
<td>pH 10.0 carb</td>
<td>3.5</td>
</tr>
<tr>
<td>3-MeLf</td>
<td>C(_6)H(_5)CH(_2)</td>
<td>pH 9.7 phos</td>
<td>3.7</td>
</tr>
<tr>
<td>3-MeLf</td>
<td>C(_6)H(_5)CH(_2)</td>
<td>pH 10.8 phos</td>
<td>2.5</td>
</tr>
<tr>
<td>3-MeLf</td>
<td>HO(_2)CH(_2)CH(_2)</td>
<td>pH 10.8 phos</td>
<td>1.8</td>
</tr>
<tr>
<td>3-MeLf</td>
<td>p-HO(_2)C(_6)H(_5)CH(_2)</td>
<td>pH 10.9 carb</td>
<td>15</td>
</tr>
</tbody>
</table>

\(^a\) (RSH) \(\gg\) (F) in all these, \(^b\) (F) = 1.0 \(\times\) \(10^{-4}\) M. \(^c\) (RSH) = 1.0 \(\times\) \(10^{-2}\) M. \(^d\) is 1 : 1 methanol : aqueous 0.02 M carbonate pH 10.3 buffer; II is 1 : 1 methanol : aqueous 0.03 M phosphate pH 11.8 buffer; the lower set of media are completely aqueous 0.1 M buffers with pH values taken before and after the reaction. \(^e\) By measuring the rate of loss of the flavin 440–450 nm absorption. Except for those runs in footnote (f) all changes were rapidly reversible upon aeration. \(^f\) These were very slow under the reaction conditions, and so are quite irreversible and may contain components due to irreversible isoalloxazine destruction. \(^g\) May be a low value due to volatility of mercaptan.
fer) and 0.79 × 10^{-4} \text{ M} 3\text{-methylumiflavin (or di-}
hydro) for both reactions with 1.0 \times 10^{-2} and
5.0 \times 10^{-2} \text{ M } 2\text{-mercaptoethanol (or its disulfide).}
These rate constants are 2.9 \times 10^{-5} \text{ sec}^{-1} \text{ and } 3.4 \\
\times 10^{-5} \text{ sec}^{-1} \text{ (and } 7.5 \times 10^{-5} \text{ sec}^{-1} \text{ and } 15.2 \\
\times 10^{-5} \text{ sec}^{-1} \text{), leading to an average equilibrium con-
stant for eqn. (8) of 0.30 at these conditions.}

\[2 \text{HOCH}_2\text{CH}_2\text{SH + 3-MeL}f \rightleftharpoons \\
\text{HOCH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2\text{OH + 3-MeL}f\text{H}_2 \] (8)

Most recently, we have chosen to do detailed work
with \text{p-carboxybenzyl mercaptan}, which is quite
water soluble as the disulfide also. The overall
kinetic behavior is rather complicated, and is cur-
rently being pursued.

C. Alkylphenone enolates

In a study of the reactions of a range of anions
derived from carbon acids\textsuperscript{7}, we noted that propi-
ophenone enolate underwent an immediate reaction
with flavins and isoalloxazines. This study has been
continued, and we here report on the reaction with
related enolates.

Upon anaerobic addition of acetophenone or buty-
rophenone sodium enolate (prepared by the action
of sodium hydride in DMSO on excess ketone) to
3-benzyl-10-phenylisoalloxazine (2 \times 10^{-3} \text{ M enolate,}
1 \times 10^{-3} \text{ M isoalloxazine in DMF}) the 437 nm ab-
sorbance is completely lost, and the visible spec-
trum is that of the \text{BPI radical anion (BPI}\textsuperscript{−})\textsuperscript{7} plus
a small absorbance between 600 and 700 nm. We have
quantitated the BPI\textsuperscript{−} spectrum by the procedure of
\text{EHRENBERG et al.}\textsuperscript{8}, and calculate a 50\% yield of
BPI\textsuperscript{−} (this value obtains over a very wide range
of enolate and BPI concentrations). Esr shows the
expected nine-line spectrum, and quantitation here
also shows a 50\% yield of spins. If one allows the
solution to sit anaerobically, or when using higher
concentrations of enolate, the long wavelength ab-
sorption grows with time. Upon aeration, the rad-
cial anion peaks are immediately lost and an amount
of 437 nm peak (oxidized BPI) corresponding to
the BPI\textsuperscript{−} appears. The 600 – 700 peak is stable
to air for hours, but after longer periods it is lost
with return of oxidized BPI. A typical series of
spectra are given in Fig. 1. Anaerobic aqueous HCl
(but not water or base) leads to immediate loss of
the long wavelength species, and this change is not
reversible upon adding base.

The material absorbing at long wavelength was
isolated for both acetophenone and butyrophenone
(but still in impure form) from THF-pentane, and
by mass spec and microanalysis seems to be an ad-
duct of enolate and BPI. If it is generated in the
reaction and then the entire mixture photolyzed for
several hours, all visible peaks are bleached. Upon
admission of air, BPI and the long wavelength materi-
al return, showing that the latter is also photo-
reducible (Fig. 2). We could not obtain evidence for
this material being a complex by dilution experi-
ments (it obeys \text{B e e r}'s Law from 10^{-3} to 10^{-6} \text{ M})
and it could not be generated by adding ketone or
enolate to any partially or fully reduced BPI solu-
tion.

The spectrum of the butyrophenone adduct has a
broad peak with one maximum at 620 nm (Figs. 1
and 2), while that from acetophenone has two
maxima, at 630 and 685 nm (Fig. 3). These spectra
are very similar to that of the 5-alkylated neutral
radical\textsuperscript{9}, but we have not been able to obtain esr
spectra. From attempts at nmr, there seems to be a
paramagnetic species in solutions of these blue com-
pounds, but at this time we cannot resolve the issue.

Upon acidification and prolonged aeration of the

![Fig. 1. Reaction of isoalloxazine with butyrophenone enolate.](image-url)
acetophenone adduct a total amount of 80% of the initial BPI is recovered (Fig. 3). This is probably complete recovery of BPI from the major reaction, the remainder lost in side reactions with base *. We have observed ketone-derived products from this set of reactions, these currently being under investigation.

At this stage, we tentatively formulate the process described here as:

\[
\begin{align*}
\text{O}^+ & \\
\text{BPI} + \text{ArC} = \text{CHR} & \rightarrow \text{HA} - \text{BPI}^- \\
& \quad \quad \quad \text{(HA')}
\end{align*}
\]

\[
\begin{align*}
\text{HA} - \text{BPI}^- + \text{BPI} & \rightarrow \text{HA} - \text{BPI}^- + \text{BPI}^- \\
\text{HA} - \text{BPI}^- & \rightarrow \text{BPI}^- + \text{AH}^- \\
\text{BPI}^- + \text{AH}^- & \rightarrow \text{BPI} + \text{A}
\end{align*}
\]

where BPI–AH– (the second adduct) is the blue species, and possibly a dimerized 5-alkylated radical. Its formation in step (11) is slow, and occurs by rearrangement of the initial adduct radical (designated HA–BPI–). In turn, this (non-absorbing) species is formed from an adduct (at position 4a or 10a) of HA– and BPI (9) by disproportionation with starting BPI to give 50% BPI– (10) 9. Steps (9) and (10) would be very rapid and quantitative, while step (11) is slow.

This scheme is the minimum one in accord with our data. The reaction describes a base catalyzed substitution and eventual oxidation of an organic compound, very similar to substrates of a number of flavoenzymes, by a flavin analogue.

In conclusion, we would like to point out that the three dark reactions discussed here represent three different kinds of flavin mediated oxidation of organic compounds 6:

1. Electron transfer: as exemplified by the quinones.

2. Substitution-elimination: in which the original nucleophile is rapidly lost without its electrons, leaving 1,5-dihydroflavin as the product. Mercaptans probably proceed in this manner.
3. Substitution: where the nucleophile is attached to the ring system in a stable manner, only coming off upon additional catalysis.

The modes of reaction of substituted reduced flavins have been pointed out previously — and we see here each of the kinds of behavior. In many systems, however, the basic processes are followed or accompanied by further reactions of the isalloxazines. In a flavoenzyme, however, the catalysis can direct the coenzyme and substrate along just one path — leading to a much higher specificity and single product.

**Experimental**

The techniques have been described elsewhere. Materials were commercial reagent grade, purified by appropriate methods before use or synthesized in this laboratory. Enolate adducts were isolated by stripping off solvent from reaction mixtures, triturating the residue with THF, decanting the solution and adding pentane to it. The precipitate was recrystallized from THF/pentane several times. The cells used for absorption spectra varied from 0.1 to 10 cm fused cells with Schlenk adapters for anaerobic studies under argon.

This research was supported in part by the U.S. Public Health Service (GM-15100) and by the Research Corporation. C. K. was an Environmental Sciences Trainee at UCR. The authors gratefully acknowledge this support.

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6. G. T. C. Bruce, L. Main, S. Smith, and P. Y. Bruce, Amer. chem. Soc. 93, 7326 [1971].
11. For a discussion of substituted reduced flavins and their chemistry, see P. Hemmerich, S. Ghisla, U. Hartmann, and F. Müller, in H. Kamin (Ed.), "Flavins and Flavoproteins", p. 83, University Park Press, Baltimore; and ref. 9b.

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