Absorption Spectrum of Flavin Mononucleotide Semiquinone

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The existence of a semiquinone as an intermediary type between the oxidized and reduced forms of flavin has been known (1—3). This semiquinone type has been studied spectroscopically on partial reduction of the flavins by reducing agents (1, 2) or by X-ray irradiation in the presence of ethanol (3), and a further work on this subject was performed by Beinert (4). These results revealed that flavin semiquinone exists in an equilibrium state with oxidized and reduced forms, and also, that the semiquinone itself takes at least two forms depending on the pH; it is green at pH>1, and is red at pH<1. Under those conditions, the absorption spectra are those of the equilibrium mixture of the oxidized, reduced, and semiquinone forms. Since the absorption maximum and extinction coefficient of the semiquinone itself has not yet been determined, a quantitative analysis of flavin semiquinone was not possible. In the present work, the absorption spectrum of the red semiquinone was studied with flavin mononucleotide in hydrochloric acid solution and its extinction coefficient was determined.

EXPERIMENTAL

FMN was a commercial sample, and found to be chromatographically pure. Concentration of the FMN stock solution was determined spectrophotometrically by using the molecular extinction coefficient of $12.2 \times 10^3 M^{-1} cm^{-1}$ at 450 m$\mu$ and pH 7.0 (5). Spectral measurements of FMN solution containing the semiquinone form were made by the use of Hitachi photoelectric spectrophotometer model EPU-2A under anaerobic condition in the presence of constant concentrations of the total FMN and hydrochloric acid, since the absorption spectrum of the oxidized flavin shows a marked change with pH as was shown by Michaelis (2).

Reduced FMN—FMN was reduced with hydrochloric acid and zinc powder in nitrogen atmosphere.

Oxidized FMN—The reduced FMN solution thus prepared was brought back to the oxidized form by shaking with air and then nitrogen gas was bubbled to remove dissolved oxygen completely. The oxidized and reduced FMN solutions were each stored in a vessel with a rubber stopper, in nitrogen atmosphere.

FMN Semiquinone—The reduced and oxidized FMN solutions thus prepared were mixed in order to make FMN semiquinone. The equilibrium between these components was attained rapidly. The mixing was carried out in an anaerobic vessel (9) which was provided with a jointed cell of 10 mm. optical path adaptable to the cell chamber of the spectrophotometer. Injection syringes were used to transfer the aliquots and the ratio of oxidized to reduced form mixed was recorded. After each measurement, the pH of the solution was checked with a glass electrode pH-meter model HM-5A of the Toa Electronic Co. All the measurements were made at room temperature.

RESULTS AND DISCUSSION

Since it was stated by Michaelis (2) that the absorption spectrum of the oxidized flavin changes with pH, the spectral change was re-examined around the particular pH used in this work. The results obtained are presented in Fig. 1. In this figure, isosbestic points were observed at 423 and 360 m$\mu$, and the extinction coefficient at 423 m$\mu$ was found to be $9.8 \times 10^3 M^{-1} cm^{-1}$. Fig. 2 shows the absorption spectra of the oxidized FMN in the presence and the absence of zinc chloride. Contrary to the suggestions made by Beinert (4), zinc ion has no effect on the spectrum as may be seen in Fig. 2.
In Fig. 3, the absorption spectra of FMN of oxidized, reduced, and at some intermediary oxidation levels at pH - 0.4 are recorded. By the use of an isosbestic point of reduced (R) and oxidized (T) FMN at 520 μm, the increment of optical density at this wavelength (ΔO.D.520) is assumed according to Beer's law to be proportional to the amount of semiquinone formed

\[ \Delta \text{O.D.}_{520} = (\varepsilon_S - \varepsilon_T)[S] \] (1)

where \( \varepsilon_S \) and \( \varepsilon_T \) are molecular extinction coefficients of semiquinone and oxidized form of FMN at 520 μm, and [S] the concentration of FMN semiquinone. Since the semiquinone is formed by the equilibrium \( T + R \xrightleftharpoons{2S} \), and the total concentration of FMN was kept constant in all measurements, there exist the following relationships

\[ [T] + [S] + [R] = [T_0] + [R_0] = \text{const.} \] (2)

\[ [T] = [T_0] - 1/2[S] \] (3)

\[ [R] = [R_0] - 1/2[S] \] (4)

\[ K = [S]^2/[T][R] \] (5)

where \( [T_0] \) and \( [R_0] \) are the initial concentrations of \( T \) and \( R \), and \( K \) the "effective formation constant" for semiquinone as defined by Michaelis (7).

In Fig. 4, \( \Delta \text{O.D.}_{520} \) is plotted against the relative value of \( [R_0] \). From the equations given above, the tangent of the curve in Fig. 4 at \( [R_0] = 0 \) is represented by the formula

\[ \Delta \text{O.D.}_{520} = 2(\varepsilon_S - \varepsilon_T)[R_0] \] (6)

so that the value of \( (\varepsilon_S - \varepsilon_T) \) can be obtained graphically. By using the value of \( \varepsilon_S \) thus obtained, [S] can readily be calculated at each \([R_0]\). Since the amount of semiquinone is maximal at \([R_0] = 0.50 \% \), it was found that the \([S]_{\text{max}} \) is 70 per cent of the total FMN concentration at this pH, and the value of

* Under the said condition, the exact value of the hydrogen ion concentration could not be determined titrimetrically owing to the presence of zinc ion in the mixture. The exact experimental condition can be reproduced by checking the absorption spectrum of the oxidized FMN as shown in Fig. 1. In the experiment shown in Fig. 3, the hydrochloric acid concentration was estimated as 2.5 N.
FIG. 3. Absorption spectra of oxidized, reduced, and intermediary oxidation levels of FMN.

FMN concentration, $3.2 \times 10^{-5} M$, pH, -0.4. In curve I, FMN is reduced 21.0 per cent and in curve II, FMN is reduced 74.3 per cent.

K is 22. Theoretical curve of the $\Delta O.D.520$ to $[R_0]$ relationship which was calculated from the K-value obtained, was found to fit completely with the experimental curve. By using the value of K thus obtained, semiquinone spectrum was obtained from calculation on the intermediary spectra shown in Fig. 3 and the results obtained are given in Fig. 5. There is a good agreement in the curves obtained from $[R_0]=21.0\%$ and 74.3\%. As may be seen in Fig. 5, two absorption maxima were found at 350 and 490 m\(\mu\) within the range from 340 to 600 m\(\mu\) and the extinction coefficients were found to be $10.0 \times 10^3$ and $9.4 \times 10^3 M^{-1} cm^{-1}$, respectively.

FIG. 4. The plot of $\Delta O.D.520$ against relative value of $[R_0]$. The straight line represents tangent of the curve at $[R_0]=0$. FMN concentration is $3.2 \times 10^{-5} M$, pH -0.4.

Fig. 5. Semiquinone spectrum of FMN at pH -0.4. This spectrum was calculated by using the “effective semiquinone formation constant”, K, obtained from Fig. 4 and the intermediary spectra of FMN presented in Fig. 3. Obtained from the curve I in Fig. 3. Obtained from the curve II in the same figure.

**Table I**

<table>
<thead>
<tr>
<th>pH of reaction mixture</th>
<th>Ratio of semiquinone concentration to that of total flavin at $[R_0] = 50%$.</th>
<th>Effective semiquinone formation constant (K)</th>
<th>Wavelengths of absorption maxima of semiquinone (m(\mu))</th>
<th>Molecular extinction coefficient ($M^{-1} cm^{-1}$)</th>
<th>Total flavin concentration used (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-0.4)</td>
<td>70</td>
<td>22</td>
<td>350 490</td>
<td>$10.0 \times 10^3$ 9.4 $\times 10^3$</td>
<td>$3.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.1</td>
<td>58</td>
<td>7.5</td>
<td>350 490</td>
<td>$9.3 \times 10^3$ 9.6 $\times 10^3$</td>
<td>$3.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.5</td>
<td>51</td>
<td>4.4</td>
<td>350 490</td>
<td>$9.1 \times 10^3$ 9.2 $\times 10^3$</td>
<td>$3.5 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
As may be seen in Table I, the values of formation constant, $K$, increased with the increasing hydrogen ion concentration within the pH range tested. However, the wavelengths of absorption maxima and the values of molecular extinction coefficients at these wavelengths were the same, within the experimental error, irrespective of the hydrogen ion concentration of the media.

Beinert (4) observed an increase of absorption in the near-infrared region, accompanied with the increase at about 500 m$\mu$, upon partial reduction of FMN. This absorption increment in the near-infrared was attributed to the formation of a semiquinone dimer. In the present work, much lower concentration of FMN was employed and no increase of absorption in the near-infrared was observed, but the possibility can not be completely ruled out that there is a formation of a trace of semiquinone dimer as pointed out by Beinert. Taking this possibility into consideration, the extinction coefficient obtained here may be a minimum value and the true extinction might be slightly larger than this value.

**SUMMARY**

Absorption spectrum of partly reduced flavin mononucleotide in hydrochloric acid solution was analyzed and the value of the "effective semiquinone formation constant" ($K$) was estimated by a graphical method. By using the value of $K$, absorption spectrum of semiquinone was determined. It was found that flavin mononucleotide semiquinone has two absorption maxima, at 350 and 490 m$\mu$, and that the extinction coefficients at these wavelengths are $10.0 \times 10^3$ (350 m$\mu$) and $9.4 \times 10^3 M^{-1} cm^{-1}$ (490 m$\mu$), respectively.

Although the $K$-value increased with the increasing hydrogen ion concentration, the absorption spectrum of FMN semiquinone was the same irrespective of the hydrogen ion concentration within the pH range tested.

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**REFERENCES**