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MOLECULAR COMPLEXES OF FLAVINS AND PHENOLS. I. ABSORPTION

SPECTRA AND PROPERTIES IN SOLUTION**

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SUMMARY

We have found that alloxazine and isoalloxazine derivatives such as lumichrome, FMN and riboflavin yield highly colored solutions in strong acid with electron-rich phenols due to the formation of 1:1 charge transfer complexes between the phenol and the monoprotonated flavin. With very good donors, e.g., 1,4-naphthalenediol or trimethylhydroquinone, similar complexes also form in neutral solution. The position of the charge transfer absorption band, in solution and in crystals of the complexes, varies with the donor properties of the phenol. EPR studies indicate the presence in acidic solution of low concentrations of the flavin semiquinone.

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INTRODUCTION

The formation of molecular complexes in neutral solutions of FMN, FAD, and riboflavin and various donors, including several phenols, has been observed by numerous workers (1-10). Although there has been speculation regarding the possible biological function of such complexes, few quantitative studies of their properties have been reported.

We have found that many phenolic compounds form rather strong complexes with alloxazines and isoalloxazines in acidic solution. Furthermore, several of the phenols, which may be expected to be especially good electron donors, have been observed to produce color changes in neutral solution with FMN indicating complex formation. These observations may be of interest in view of recent reports implicating tyrosine in the binding of flavin to protein (11, 12).

Studies of complexing of indole and pyridinium ion in model compounds (13), and a comparison of complex spectra with enzyme spectra, have suggested that the binding of NAD^+ to the protein of glyceraldehyde-3-phosphate dehydrogenase may be due in part to charge transfer complexing. Spectral data suggesting that the complexes studied in the present work are also of the charge transfer type may thus be especially significant.

As an additional impetus to studies of flavin-phenol interactions, redox reactions involving reduced flavin and quinones such as coenzyme Q and vitamin K are thought to be involved in certain metabolic processes, notably in mitochondrial electron trans-

port. However, the instability of reduced flavin under aerobic conditions makes impractical a study of complex formation involving the process which is the reverse of the one investigated here.

EXPERIMENTAL

Materials

Riboflavin and FMN were obtained from the California Corporation for Biochemical Research and were used without further purification. The procedures employed in the synthesis of lumiflavin and 9-methylisoalloxazine have been described previously (15). 1,4-Naphthalenediol and 1,2-naphthalenediol were prepared by snaking chloroform solutions of the corresponding quinones with saturated aqueous sodium dithionite (16, 17). The resulting diols were recrystallized from water. Hydroquinone and pyrogallol were reagent grade, obtained from Matheson, Coleman and Bell and Mallinckrodt, respectively, and were not further purified. Other phenol derivatives were obtained from Aldrich and Eastman and in most instances were recrystallized from suitable solvents.

Formation of Complex Crystals

Crystals of the 9-methylisoalloxazine complexes could be obtained by preparing a saturated solution of 9-methylisoalloxazine in 12 N hydrochloric acid. An equal number of millimoles of the phenol were dissolved in an equal volume of ethanol. The two solutions were then mixed. Crystals formed after the mixture had been permitted to stand for a few hours. In some cases it was necessary to permit the solution to evaporate slowly before crystal formation would occur.

Large single crystals of the riboflavin-hydroquinone complex could be grown by permitting the slow evaporation of a 50% ethanol-50% 12 N HCl solution containing stoichiometric (1:1) quantities of riboflavin and hydroquinone. The solution was ideally prepared by dissolving smaller crystals of the complex from a previous batch. Concentrated hydrochloric acid alone was also found to be a suitable solvent for the growth of riboflavin-hydroquinone crystals.

Microcrystals used for the measurement of the spectra of complexes in the solid state were prepared by grinding larger crystals or by inducing rapid crystallization from supersaturated solution by scratching the walls of the vessel.

Crystals of lumiflavin and riboflavin complexes were grown from neutral solution by adding enough of the phenol to a boiling suspension of the flavin in a water-acetone mixture to take the flavin into solution. Crystals of the complex formed upon slow cooling of the solution.

Optical Spectra

Visible and near infrared spectra were obtained with the use of Cary Model 14, Cary Model 11 and Zeiss PMQ II spectrophotometers.

Two techniques, yielding essentially identical results, were employed to obtain the approximate visible spectra of riboflavin complexes in acid solution. In the first method, extremely high phenol concentrations, e.g., 6 N HCl saturated with pyrogallol, were used, so that almost all of the flavin was present in the form

of complex. In an alternate method, apparent stability constants obtained with the use of a generalization of the Scott form of the Benesi-Hildebrand equation (18) (details and data to be published later) were employed to estimate the concentration of free flavin in solutions of complexes. Solutions containing the calculated flavin concentrations were then used as blanks during the spectral measurements. The difference spectra obtained in this manner should thus be due, to a first approximation, only to the absorption of the complexes. Phenol concentrations were selected such that the flavin:complex ratio was 1:1.

Spectra of suspensions of crystalline complexes were obtained by the method of Shibata (19). Suspensions of the solids in mineral oil were centrifuged briefly to remove large particles, then were transferred to 0.1 cm Cary cells. Pieces of opal glass were positioned between the sample and reference cells and the respective phototubes. Alternatively, the sample and reference cells were placed in "integrating boxes" with rough white interiors and opaque baffles between the cells and the phototubes, so that only scattered light could reach the phototubes.

Electron paramagnetic resonance (EPR) spectra

EPR spectra were recorded in derivative presentation using a Varian V-4501 spectrometer equipped with 100 kc modulation. Spin concentrations were assumed to be proportional to signal width squared times peak-to-peak height. An aqueous solution of manganous chloride was employed as a spin concentration standard. For sample illumination, a 500 watt tungsten filament projection

lamp was employed. The beam was filtered through a water-cooled heat filter to diminish heating due to infrared radiation, and focused onto the sample cell through slits in the microwave cavity by means of a series of glass lenses.

RESULTS AND DISCUSSION

When alloxazine or its derivatives such as lumichrome, or isoalloxazine derivatives such as riboflavin, FMI or lumiflavin are placed in strongly acidic solution (greater than 1 N HCl) with phenol derivatives possessing high pi electron densities, an intense color usually develops immediately. The color may range from red (e.g., resorcinol, 2,3-naphthalenediol) through brown (e.g., hydroquinone, p-dimethoxybenzene, 4,4'-dihydroxydiphenyl) to green (e.g., 1,4-naphthalenediol). Phenols substituted predominantly with electron-withdrawing groups, such as 2,4-dinitroanisole or tetrachlorohydroquinone, display no such color reaction. Both tyrosine and phenol yield only a faint red coloration. Furthermore, with the exception of indole and its derivatives such as tryptophan, which give brown solutions with flavins in acid, only isocyclic aromatics have shown such color formation. For example, purine and pyrimidine derivatives, which on the basis of spectral changes and fluorescence quenching are believed to complex with FMI in neutral solution (2), do not give the characteristic color reaction with flavins in acid.

* Among the compounds found to give no color reaction with riboflavin in acidic solution are the following: adenylic acid, caffeine, cysteine, histidine, ascorbic acid, glucose, 8-hydroxyquinoline, 2,6-di-t-butylphenol.

The stability constants for the isonalloxazine complexes in acid are of the order of 10 liter/mole. The details of these measurements will be published shortly.

It has been possible to isolate, in crystalline form from acid solution, complexes of riboflavin, lumiflavin and 9-methylisonalloxazine with a number of phenols. The crystals seem invariably to be monoclinic. Those containing naphthalene derivatives have the form of long needles while those containing benzene derivatives are more symmetrical. The crystals are very intensely colored due to the presence of a broad absorption band in the visible region, the significance of which will be discussed below.

Optical spectra

The visible absorption spectra, in 6 N hydrochloric acid, of many of the flavin complexes resemble that of the monoprotonated form of riboflavin. The absorption with a maximum at 385 $m\mu$, however, appears to be somewhat attenuated and broadened in the complex (Fig. 1). The degree of broadening into the green region of the spectrum was found to vary with the phenol employed. In these complexes, there is no infrared absorption between 800 and 3000 $m\mu$. It has not been possible to study the spectra of the complexes in the ultraviolet region.

The spectra of the 1,4-naphthalenediol, 1,2-naphthalenediol and 1,7-naphthalenediol complexes, whose solutions are green, display an additional absorption maximum (Fig. 2). The positions of the new maximum for the three complexes are approximately 625 $m\mu$, 550 $m\mu$ and 550 $m\mu$, respectively. Spectra of solutions of

9-methylisoalloxazine with trimethylhydroquinone exhibit a distinct shoulder near 550 $m\mu$.

Spectra of suspensions of powdered complex crystals invariably display two absorption bands in the visible region (Fig. 3). The more intense absorption, with a maximum near 400 $m\mu$, is also characteristic of crystalline flavin hydrochloride, and is undoubtedly due to a broadening of the 385 $m\mu$ band of the protonated flavin. The position and shape of the broad long wavelength band is dependent upon the phenolic compound involved. Such behavior is known to be characteristic of charge transfer bands (14).

We are therefore led to believe that the broad absorption bands of the solid complexes, and the long wavelength bands of the naphthalenediol solutions, are due to charge transfer absorptions. A red shift of about 125 $m\mu$ was observed to have occurred in the position of the 1,4-naphthalenediol band during the transition from solution to solid (see Figs. 2 and 3). If the charge transfer bands of the other complexes undergo commensurate shifts during this transition, their maxima in solution must occur near that of the 385 $m\mu$ flavin band, and would largely account for the observed broadening of the latter.

pH Dependence of complex formation

As may be seen from Fig. 4, the appearance of complex in FMN-hydroquinone solutions coincides approximately with the disappearance of unprotonated FMN as the acidity of the solution is increased. Similar results have been obtained with the FMN-pyrogallol system and the riboflavin-pyrogallol system. In each

case, the pK for complex formation falls near 0.0, which is close to the literature value (21) for the acid dissociation constant for riboflavin. It is thus concluded that flavin exists in the complexes as the monoprotinated species. This conclusion is consistent with solution spectra (Fig. 1) and with the results of elemental analyses of crystalline complexes (20).

Stoichiometry in solution

If the equation describing complex formation may be written $aP + bF = P_aF_b$, where P represents phenol, F represents flavin and a and b appropriate integers, then

$$\frac{(P_aF_b)}{(P)^a (F)^b} = K,$$

where K is the stability constant of the complex. Then

$$\log (P_aF_b) = a \log (P) + b \log (F) + \log K.$$

If Beers law is assumed to be obeyed, a number proportional to (P_aF_b) may be estimated from the optical density of solutions in a region where neither component absorbs, and a and b may be evaluated from an appropriate log-log plot. This is done in Fig. 5 for the riboflavin-hydroquinone complex. From the slopes it is seen that both coefficients are equal to one. Similar results have been obtained for the riboflavin-pyrogallol, FMN-pyrogallol and 9-methylisoalloxazine-catechol complexes. One-to-one stoichiometry has also been established for several complexes in the crystalline state (20).

Electron paramagnetic resonance spectra of solutions

Solutions of most of the complexes in strong acid display a weak EPR absorption. When the isoalloxazine derivative is one for which a well-resolved semiquinone EPR spectrum can be obtained (15), the hyperfine structure of the complex solution spectrum is found to be identical to that of the semiquinone of the isoalloxazine. No signal due to a radical derived from the phenolic species could be observed.

Semiquinone concentrations were found from the intensity of the EPR absorption to be of the order of one per cent of the total flavin concentration.

The dependence of spin concentration upon pH and upon flavin and phenol concentrations is shown in Figs. 6 and 7. From the pH dependence it may be seen that the charge transfer complex studied by optical methods is not directly responsible for the EPR absorption, although the stoichiometry of the radical formation process is 1:1. It thus seems reasonable to suppose that the semiquinone is formed by complete electron transfer from phenol to flavin, and that the phenol radicals thus generated, being unstable in acid solution, immediately disproportionate and so do not contribute to the EPR absorption.

Illumination with white light was found to evoke a reversible increase (time constant of the order of minutes) in spin concentration for every system studied, presumably due to a potentiation of the dark electron transfer reaction. Acidic solutions of riboflavin and tryptophan display no EPR absorption in the dark, but a rather strong flavin semiquinone signal appears upon illumination.

It is interesting to note that the spin concentration in solution is of the same order of magnitude as that which may be achieved in crystals of the complexes (20). However, illumination induces no measurable increase in the spin concentration of crystals (although it does increase the number of charge carriers). It is hoped that further studies of the behavior of the complexes in solution will shed more light upon the nature of charge-carrier formation and migration in the solid.

Complexes in neutral solution

Several phenols (e.g., all of the naphthalenediols, trimethylhydroquinone and 4-methylcatechol) have been observed to readily form complexes with flavins in neutral solution. The absorption spectra of these solutions are qualitatively similar to the FMI-phenol spectrum presented by Yagi *et al.* (22). However, the diminution and apparent red shift of the 373 m μ and 445 m μ bands are much more pronounced in the spectra we have observed, and the spectra are characterized by broad tails extending far into the green region.

Furthermore, we have found that the amount of red shift of the complex spectra with respect to the flavin spectrum is significantly greater in water than in water-ethanol mixtures. This would be expected for a charge transfer type complex.

We have been able to isolate in crystalline form complexes of lumiflavin with 1,4-naphthalenediol, 1,7-naphthalenediol, 2,3-naphthalenediol, trimethylhydroquinone and 4-methylcatechol, and of riboflavin with 2,3-naphthalenediol. The visible spectra of

suspensions of these crystals (Fig. 8) include broad, structureless bands in the visible region which are characteristic of neither of the components. As was the case with the acidic complexes, the positions of these bands depend upon the nature of the phenol.

The presence and character of these bands, coupled with the striking similarity between the spectra of the acidic complexes and those of the neutral complexes, both in solution and in the solid state, strongly suggest that charge transfer is involved in the formation of flavin-phenol complexes in neutral as well as in acidic solution. Thus, it would appear that protonation of the flavin merely serves to increase its acceptor ability, resulting in a red shift of the charge transfer band of complexes of which it is a component.

Crystals of the lumiflavin complexes exhibit weak EPR absorption. The shape of the signal is suggestive of two superimposed absorptions or of unresolved hyperfine structure.

CONCLUSION

The ability of phenolic compounds to form charge transfer complexes with flavins provides support for an interpretation of the binding of flavin to protein (11, 12) which is based upon this property. Interactions of this sort would certainly be expected to influence the redox properties of the flavin and thus may play a role in the catalytic process.

Similarly, charge transfer reactions may be involved in the electron transfer from reduced flavin to quinones, inasmuch as it has been shown that donor-acceptor complexes can be intermediates in radical-ion formation (23).

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Fig 1

Fig 4

Fig 6

Fig 7

Fig 8