THE FLAVIN OF CHROMATIUM CYTOCHROME c-552

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SUMMARY. A flavin obtained from Chromatium cytochrome c-552 has been purified and characterized with respect to its neutral and acid absorption spectra, fluorescence-pH relationship, and behavior on partition and molecular exclusion chromatography. The chromatographic results are consistent with a flavin adenine dinucleotide type structure. Neutral and acid absorption spectra taken after degradation of the flavin to the riboflavin level differ in the near UV from those of riboflavin and are similar to those of flavins modified in the dimethylisoalloxazine ring system, such as the histidyl-riboflavin obtained from mammalian succinate dehydrogenase. However, cytochrome c-552 flavin when at the riboflavin level and histidyl-riboflavin are quite dissimilar when compared fluorometrically and by molecular exclusion chromatography.

Cytochrome c-552 as purified from Chromatium has been shown to contain a firmly bound flavin prosthetic group (1). The flavin is released on treatment of cytochrome c-552 with a saturated urea solution in the cold and when freed of urea, it exhibits a neutral absorption spectrum similar to that of FAD except for a 20 nm blue-shift in the maximum of the near ultraviolet band (2). The similarity of the absorption spectrum to that of the succinate dehydrogenase flavin peptide raised the possibility of a covalent link joining the flavin and the cytochrome protein (2). On the other hand, lability of the flavin-protein link in concentrated urea solution is not characteristic of the succinate dehydrogenase flavin (3). In view of this unusual combination of properties, the further characterization of the cytochrome c-552 flavin has been carried out.

MATERIALS AND METHODS. Chromatium strain D was grown in one liter prescription bottles on the modified (4) "heterotrophic" medium of Fuller (5). Cells were harvested by centrifugation and stored as a frozen paste at -20°. Cytochrome c-552 was isolated and purified through the first DEAE-cellulose elution

according to the procedure of Bartsch (6). Flavin was dissociated in cold, saturated urea (2) and freed of heme protein by passage through a Sephadex G-25 column equilibrated with saturated urea. The flavin-containing fractions were combined and the urea removed by passage through a Sephadex G-15 column equilibrated with 0.1 M acetic acid. The flavin-containing fractions were again combined, dried on a rotary evaporator, and stored in the dark at -20°. This material is hereafter referred to as "c-552 flavin."

Hydrolysis of the pyrophosphate linkage in flavin adenine dinucleotides was carried out in 10% trichloracetic acid (TCA) at 38° for 4 hours (7). TCA was removed by extraction into ether. The phosphate ester bond in flavin mononucleotides was cleaved by incubation at room temperature for 10-12 hours in 0.5 M acetate buffer, pH 5.0, containing 1 mg/ml potato acid phosphatase (Sigma, Type II). The reaction was stopped by heating the solution in a boiling water bath for 10 min. Precipitated protein was removed by centrifugation and the supernatant then eluted from a Sephadex G-15 column.

Paper chromatography of flavins was carried out in descending fashion in the dark at 36° using the upper layer of n-butanol:acetic acid:water (4:1:5 v/v) as the developer (8).

The riboflavin peptide used for molecular exclusion chromatography was purified from a tryptic-chymotryptic digest of beef heart mitochondrial membranes (9). Histidyl-riboflavin was obtained by acid hydrolysis of the flavin peptide (10) and purification by cation exchange chromatography (9).

RESULTS AND DISCUSSION. In Table 1 it can be seen that c-552 flavin exhibits the same R_f value as authentic FAD when compared by paper chromatography. In Figure 1 the c-552 flavin and FAD are seen to have the same elution volume when Sephadex G-15 chromatography was carried out. When c-552 flavin and FAD were subjected to mild acid hydrolysis, products were obtained which were indistinguishable from FMN in both chromatographic systems. On treating these products with acid phosphatase they were further altered and displayed R_f values similar to riboflavin on paper chromatography. The acid hydrolyzed,

TABLE 1

Paper Chromatography of Flavins

Sample	R_f
FAD	.05
c-552 flavin	.05
FMN	.13
FAD after TCA hydrolysis	.13
c-552 flavin after TCA hydrolysis	.13
riboflavin	.36
FAD after TCA hydrolysis and acid phosphatase treatment	.37
c-552 flavin after TCA hydrolysis and acid phosphatase treatment	.35

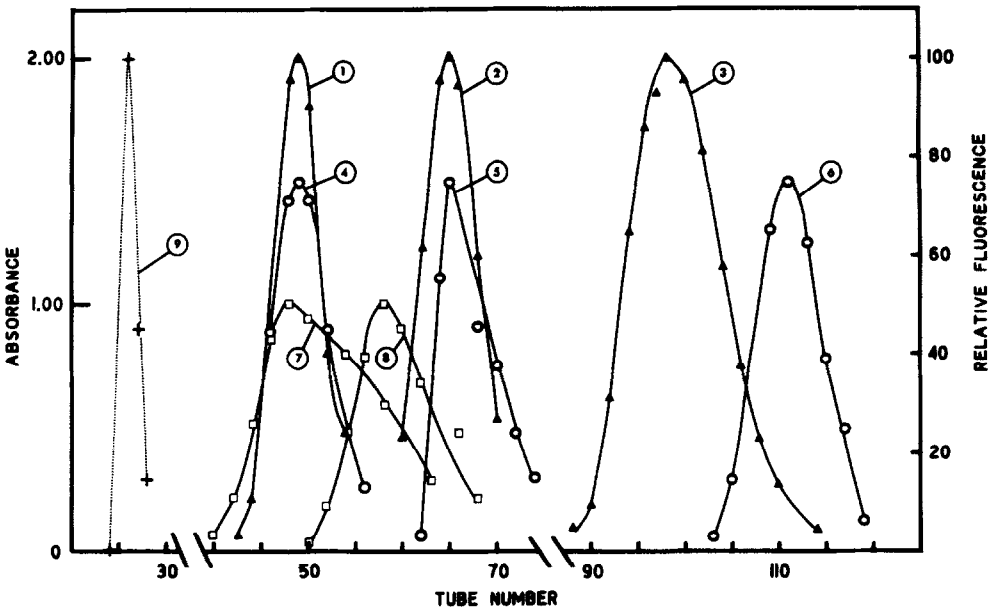

Fig. 1. Molecular exclusion chromatography of flavins. Flavins were applied to a 2 x 70 cm. column of Sephadex G-15 that had been equilibrated with 0.1 N acetic acid, eluted with 0.1 N acetic acid, and collected in 3.0 ml. fractions. c-552 flavin, ①; c-552 flavin after TCA hydrolysis, ②; c-552 flavin after TCA hydrolysis and acid phosphatase digestion, ③; FAD, ④; FMN, ⑤; riboflavin, ⑥; succinate dehydrogenase riboflavin peptide, ⑦; histidyl-riboflavin, ⑧; void volume marker: horse heart cytochrome c, ⑨.

phosphatase treated c-552 flavin was eluted from Sephadex G-15 slightly ahead of riboflavin and well after both histidyl-riboflavin and the riboflavin peptide

from succinate dehydrogenase. These observations suggest that c-552 flavin is an adenine dinucleotide. This view is further supported by the paper chromatographic identification of adenine in ether extracts of the neutralized TCA hydrolysate and by the observation of a maximum at pH 2.8-2.9 in the fluorescence intensity-pH relationship for c-552 flavin as isolated (11).

The blue shift in the near ultraviolet absorption band which is seen in the neutral c-552 flavin spectrum remains a characteristic feature in the spectra obtained after TCA hydrolysis and also after TCA hydrolysis and acid phosphatase digestion. Both neutral and acid spectra of the latter preparation are shown in Figure 2. The spectrum of this material obtained in acid solution is also distinct from the corresponding riboflavin spectrum as a consequence of a blue-shift in the absorption maximum. The similarity of the neutral and acid spectra of c-552 flavin after TCA hydrolysis and acid phosphatase treatment to those of histidyl-riboflavin from mammalian succinate dehydrogenase is apparent in Figure 2. Spectral alterations of this sort

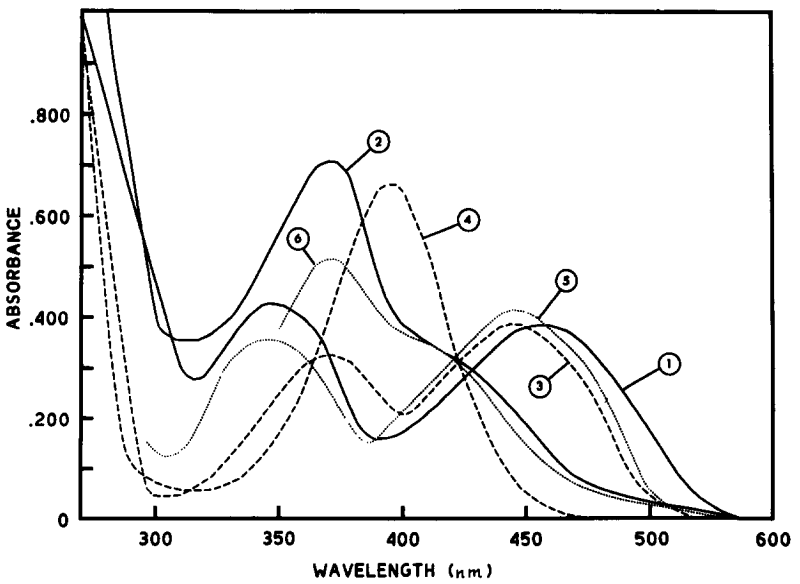

Fig. 2. Absorbance spectra of flavins. Spectra were obtained in water (neutral) or 6N HCl (acid) using a Cary 14 spectrophotometer and quartz cells of 1 cm. light path. c-552 flavin after TCA hydrolysis and acid phosphatase digestion: neutral, ① and acid, ②; riboflavin: neutral, ③ and acid, ④; histidyl-riboflavin: neutral, ⑤ and acid, ⑥. Histidyl-riboflavin spectra have been replotted for comparison from Ghisla, et al. (13).

have been described for several derivatives of the dimethylisoalloxazine system involving substitutions at the 8-methyl position (12).

The pH dependence of the fluorescence intensity of c-552 flavin after TCA hydrolysis and acid phosphatase treatment is shown in Figure 3. Corresponding data for riboflavin and histidyl-riboflavin from succinate dehydrogenase have been replotted for comparison (13). It can be seen that whereas histidyl-riboflavin undergoes a transition to a less fluorescent form with a pK of 4.5, the material derived from c-552 flavin is similar to riboflavin, showing a broad

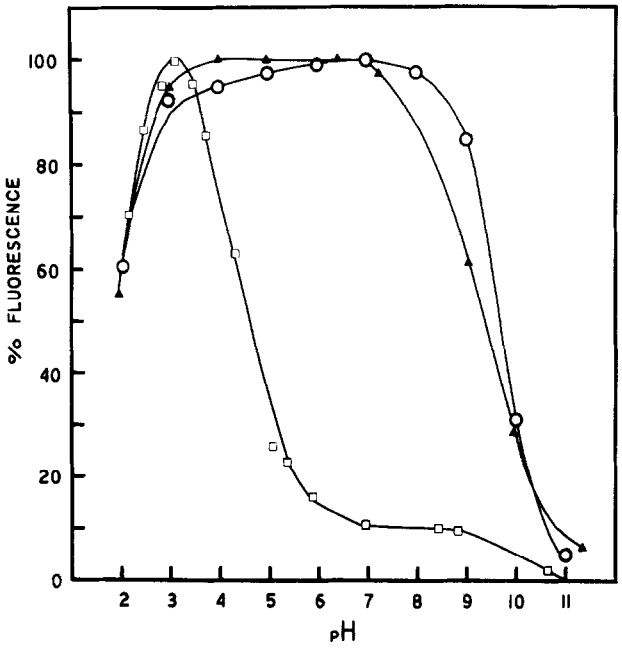

Fig. 3. pH Dependence of flavin fluorescence. c-552 flavin, ▲; histidyl-riboflavin from succinate dehydrogenase, □; and riboflavin, ○. Samples of c-552 flavin after TCA hydrolysis and acid phosphatase digestion were added to 0.03 M buffer solutions. The buffers used were: pH 1.95, KCl:HCl; pH 3.00, glycine; pH 4.00 and 4.93, acetate; pH 6.36 and 7.25, phosphate; pH 9.00 and 9.95, borate; pH 11.30, phosphate. The data for histidyl-riboflavin and riboflavin have been replotted for comparison from Ghisla, et al. (13).

region of maximal fluorescence which declines to half-maximal intensity at about pH 9.3. This finding seems to rule out a substitution of the dimethyl-isoalloxazine system by an imidazole group. A fluorescence-pH relationship with a similar broad maximum has recently been reported for the flavin of beef

liver monoamine oxidase. This flavin exhibits spectral properties very much like those of the succinate dehydrogenase flavin and, like that flavin, is released only by proteolysis of the enzyme (14).

Taken together these facts suggest that c-552 flavin is an adenine dinucleotide in which the dimethylisoalloxazine ring system is substituted in the 8-methyl position. The substituent is presumably not an imidazole group and, as judged by comparison with riboflavin and histidyl-riboflavin on molecular exclusion chromatography, it must be of relatively small size. Further characterization of this flavin is in progress.

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