IDENTIFICATION AND PROPERTIES OF 8α-[N(1)-HISTIDYL]-RIBOFLAVIN: THE FLAVIN COMPONENT OF THIAMINE DEHYDROGENASE AND β-CYCLOPIAZONATE OXIDOCYCLASE

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SUMMARY

In addition to 8α-[N(3)-histidyl]-riboflavin which had previously been characterized as the product on condensation of N_{α}-blocked histidine with 8α-bromotetraacetylriboflavin (after removal of the blocking groups), a second histidylflavin isomer is obtained in 20-25% yield of the total histidylflavin fraction. This isomer is identified as 8α-[N(1)-histidyl]-riboflavin by chemical degradation of the histidylflavin analog after alkylation of the imidazole with methyliodide. Acid hydrolysis at high temperature yields 3-methylhistidine, identified by its mobility on high voltage electrophoresis, while Zn reduction yields riboflavin, identified by thin layer chromatography. The properties of synthetic 8α-[N(1)-histidyl]-riboflavin are identical with the histidylriboflavin obtained from thiamine dehydrogenase and β-cyclopiazonate oxidocyclase in pK_a of fluorescence quenching, electrophoretic mobility, and in reduction by sodium borohydride. Thus, both the N(1) and the N(3) histidylriboflavin isomers occur in nature. The compound obtained on acid treatment of 8α-[N(3)-histidyl]-riboflavin (previously thought to be 8α-[N(1)-histidyl]-riboflavin) is shown to differ from the parent compound only in the ribityl side chain.

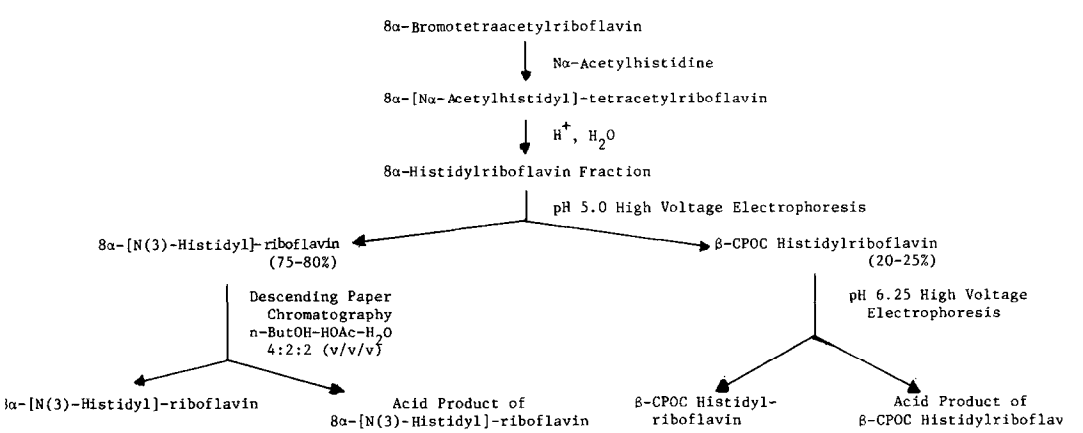
Approximately two years ago, we reported (1,2) that the covalently bound flavin components of thiamine dehydrogenase and β-cyclopiazonate oxidocyclase differed in properties from all three classes of covalently bound flavins then known and thus represented a fourth type of covalently bound flavin structure. Flavin peptides, after hydrolysis to the FMN level, derived from either enzyme can be degraded by acid hydrolysis to an 8α-histidylriboflavin which differs from 8α-[N(3)-histidyl]-riboflavin in being considerably more labile and in having a pK_a of fluorescence quenching of 5.0 to 5.1, which is some 0.6 pH units higher than that of the known compound. The two components are separable by electrophoresis at pH 5.0. Further differences subsequently discovered include the fact that the new type of histidylriboflavin yields 8-formylriboflavin on storage and is reduced by borohydride, although on subsequent exposure to oxygen the original flavin is not recovered. Neither of these properties is shown by 8α-[N(3)-histidyl]-riboflavin.

The new type of histidylriboflavin also differed in pK_a and in stability from a model compound which was then thought to be 8α-[N(1)-histidyl]-riboflavin, which had been described earlier (3) as an acid modification product of the N(3) isomer. With both of the imidazole nitrogens of the histidine seemingly excluded as the site of attachment of the 8α group of FAD it was tentatively suggested (1) that perhaps either C_2 or C_4 of the imidazole might be the anchoring point of the flavin.

In order to examine this possibility as well as other alternative structures later proposed, micromole quantities of this new type of histidyl flavin were required, more than could be obtained by isolation from enzymes. Fortunately, the compound could be obtained as a side product of the synthesis of 8α-[N(3)-histidyl]-riboflavin (1,2), amounting to some 20-25% of the total histidylriboflavin formed (Scheme 1). As in the case of 8α-[N(3)-histidyl]-riboflavin, also in the case of this product two compounds are obtained, the parent histidylriboflavin, and its acid modification product. The two may be separated by paper chromatography or by high voltage electrophoresis. This synthetic compound was in every respect identical with the histidylriboflavin isolated from thiamine dehydrogenase or β-cyclopiazonate oxidocyclase with regard to electrophoretic mobility, pK_a of fluorescence quenching, and reactivity with borohydride. Preliminary proton magnetic resonance studies at 90 MHz (in collaboration with Dr. A. Schonbrunn, Brandeis University) showed the C_2 and C_4 protons of the imidazole ring to be present, thus eliminating these positions as the site of flavin attachment (4). The same experiments seemed to indicate that the 8α carbon of the flavin possessed a single proton, suggesting

SCHEME 1

ISOLATION OF SYNTHETIC β-CPOC HISTIDYLRIBOFLAVIN



the possibility that the new histidyl flavin was an adduct of 8-formylriboflavin and histidine (4). This speculation seemed to be born out by the finding of 8-formylriboflavin as the product of hydrolysis of the new histidyl flavin (4,5).

This note presents conclusive evidence against the 8α-histidyl-8α-hydroxyriboflavin structure proposed and shows instead that the compound is 8α-[N(1)-histidyl]-riboflavin. The compound previously believed to have this structure (3) is shown to be a derivative of 8α-[N(3)-histidyl]-riboflavin, modified in the ribityl side chain on acid treatment.

MATERIALS AND METHODS

8α-Bromotetraacetylriboflavin, 8α-[N(3)-histidyl]-riboflavin and the acid modified product of 8α-[N(3)-histidyl]-riboflavin were synthesized and isolated as outlined by Walker et al. (3). The synthetic β-CPOC histidyl flavin* was isolated from the histidylriboflavin fraction by preparative high voltage electrophoresis at pH 5.0, 40 V/cm for 2 hours. Methylation and Zn cleavage were performed essentially as described (3). The method of cleavage of methylated histidylriboflavins varied with the position on the imidazole ring on which the flavin was substituted and also with the product sought. For near quantitative yield of flavin from either methylated N(1) or N(3) histidylriboflavin, Zn reduction was used but this method resulted in low yield of methylhistidines. For liberation of the latter in good yield from methylated N(1) histidylriboflavin hydrolysis in 6N HCl at 125° was required and from methylated N(3) histidylriboflavin hydrolysis in 6N HCl at 150° was required. In comparing the products from the different methylated histidylriboflavins, 150° was used. The methylhistidines were identified by high voltage electrophoresis at pH 6.25, 50 V/cm, 60 min.

Absorption spectra were obtained using a Cary 14 spectrophotometer, corrected fluorescence spectra from a Perkin Elmer MPF 3 spectrofluorometer and proton magnetic resonance spectra at 300 MHz using a Fourier transform NMR spectrometer at Varian instruments.

RESULTS AND DISCUSSION

Table I presents the results of mechanical integration of the flavin and imidazole protons from proton magnetic resonance spectra taken at 300 MHz of authentic 8α-[N(3)-histidyl]-riboflavin, synthetic β-CPOC histidyl flavin and its acid modified product. It is clear that the 8α carbon of all three compounds contains two protons, rather than one, thus eliminating the possibility that the β-CPOC histidyl flavin contains a riboflavin moiety at which the 8α carbon is at the oxidation state of a carbonyl.

Data to be published show that reductive cleavage of either the synthetic or natural β-CPOC type of histidylriboflavin by Zn in acid media, following methylation of the imidazole ring, yields authentic riboflavin in 80-90% yield. This rules out any

*β-CPOC histidyl flavin denotes the synthetic histidyl flavin with properties identical to the histidylriboflavin from β-cyclopiazonate oxidocyclase and thiamine dehydrogenase.

modification of the flavin moiety at positions other than at 8α. The proof that the imidazole attachment to the flavin was indeed at the 8α position, as in all other naturally occurring, covalently bound flavins thus far described, was obtained from electron magnetic resonance and electron-nuclear double resonance data (5). Since this seemed to rule out all possible structural alternatives, it was necessary to examine the basic assumptions used.

The structure of 8α-[N(3)-histidyl]-riboflavin had been firmly established (3) by methylation, Zn cleavage, and chromatographic identification of 1-methylhistidine as the product. However, the structure of what was correctly recognized and proven to be an acid modification product of this compound or of the naturally occurring flavin peptide of succinate dehydrogenase was deduced but not rigorously proven to be 8α-[N(1)-histidyl]-riboflavin (3). It therefore became necessary to reinvestigate the structure of the acid-modified product of 8α-[N(3)-histidyl]-riboflavin.

We have now treated the acetylated derivative of the acid modification product of 8α-[N(3)-histidyl]-riboflavin with methyl iodide to quaternize the imidazole nucleus, cleaved the histidyl flavin bond by acid hydrolysis at 150° and identified the liberated methylhistidine by high-voltage electrophoresis (Table II). The product from both 8α-[N(3)-histidyl]-riboflavin and its acid modification product was 1-methylhistidine, thus proving that in both cases the flavin is attached to the N(3) position of the imidazole ring and that the acid-modified compound is not 8α-[N(1)-histidyl]-riboflavin.

A clue to the identity of the acid-modified flavin previously thought to be 8α-[N(1)-histidyl]-riboflavin came from examination of the published (3) proton magnetic resonance spectra which shows a major difference in the ribityl protons when compared with the spectrum of 8α-[N(3)-histidyl]-riboflavin. The simplest explanation is that acid does not catalyze an N(3) to N(1) flavin migration but modifies the ribityl side

TABLE I

INTEGRATION OF FLAVIN AND IMIDAZOLE PROTONS OF SYNTHETIC HISTIDYLRIBOFLAVINS

Flavin	FL(7)-CH_3	FL(8)-CH_2	FL(7)-H	FL(9)-H	Im(4)-H	Im(2)-H
8α-[N(3)-Histidyl]-riboflavin	3.0	2.0	0.8	0.6	0.6	0.9
Synthetic β-CPOC histidylriboflavin	3.0	1.8	0.8	0.9	0.7	0.9
Acid product of β-CPOC histidyl-riboflavin	3.0	2.1	0.7	0.6	0.7	0.8

TABLE II

IDENTIFICATION OF METHYLHISTIDINE PRODUCT FROM ACID HYDROLYSIS OF METHYLATED 8α-HISTIDYLFLAVINS*

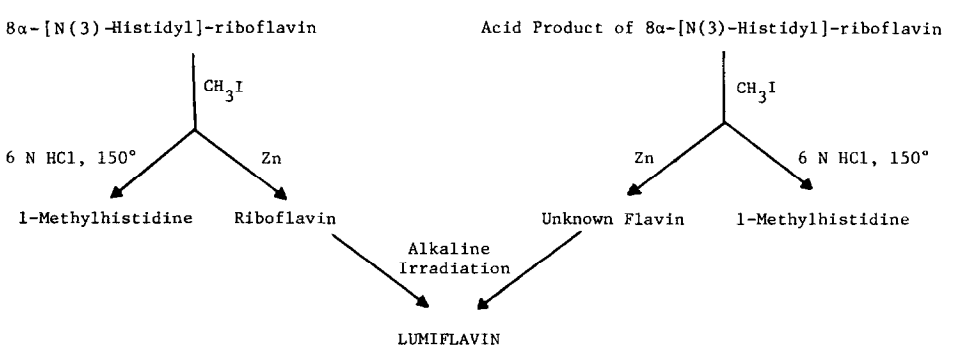
Compound	Electrophoretic mobility to anode (cm)	Identity of product
Histidine	15.1	
1-Methylhistidine	16.9	
3-Methylhistidine	14.0	
Product from 8α-[N(3)-histidyl]-riboflavin after methylation	16.9	1-Methylhistidine
Product from acid-modified 8α-[N(3)-histidyl] -riboflavin after methylation	16.9	1-Methylhistidine
Product from β-CPOC histidyl flavin after methylation	14.0	3-Methylhistidine

*The methylhistidines were located by spraying the paper with 0.2% ninhydrin in n-butanol.

chain. This would explain the finding that the flavin product from Zn cleavage of the acid-product of 8α-[N(3)-histidyl]-riboflavin yields a flavin with the same absorption, fluorescence, and EPR properties as riboflavin (the product obtained from 8α-[N(3)-histidyl]-riboflavin) but differing in mobility on thin-layer chromatography (5). Proof for ribityl side chain modification was obtained by converting the flavin product from Zn cleavage to authentic lumiflavin by alkaline irradiation (Scheme 2).

SCHEME 2

DEGRADATION OF 8α-[N(3)-HISTIDYL]-FLAVINS TO LUMIFLAVIN



Having thus established that the model compound previously thought to be 8α-[N(1)-histidyl]-riboflavin has a different structure, the possibility that the synthetic β-CPOC histidyl flavin is the N(1) isomer could once again be considered. As shown in Table II, the methylhistidine formed upon methylation and acid cleavage of synthetic β-CPOC histidyl flavin is identified as 3-methylhistidine, thus proving the flavin-histidine linkage to be at the N(1) position of the imidazole ring. Thin-layer chromatography identified the liberated flavin after Zn cleavage to be riboflavin, thus proving the structure to be 8α-[N(1)-histidyl]-riboflavin. As in the case of the acid-modified product from 8α-[N(3)-histidyl]-riboflavin, the flavin liberated from the acid product of 8α-[N(3)-histidyl]-riboflavin, the flavin liberated from the acid product of 8α-[N(1)-histidyl]-riboflavin is also modified in the ribityl side chain, since both liberated flavins show identical migration on thin-layer chromatography and are converted to authentic lumiflavin on alkaline irradiation (Scheme 2).

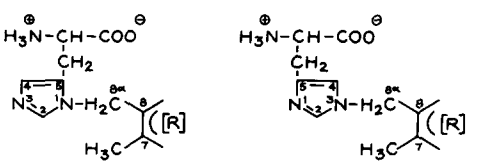


Fig. 1 Structures of: 8α-[N(1)-histidyl]-flavin (left side) and 8α-[N(1)-histidyl]-flavin (right side). R denotes rest of flavin moiety.

Fig. 1 compares the structures of the two types of 8α-histidyl-FAD occurring in nature. So far three enzymes have been shown to contain each of the two histidyl flavin isomers. A recent report from Sato's laboratory (6) clearly shows that in addition to thiamine dehydrogenase (1) and β-cyclopiazonate oxidocyclase (2), L-gulono-γ-lactone oxidase from liver microsomes contains a flavin with the properties which characterize it as 8α-[N(1)-histidyl]-FAD. The N(3) isomer, previously characterized for succinate dehydrogenase (3), has also been shown to be present in D-6-hydroxynicotine oxidase (7) and appears to be the form of the histidyl flavin in sarcosine dehydrogenase (8).

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