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Studies on Succinate Dehydrogenase

8α-Histidyl-FAD as the Active Center of Succinate Dehydrogenase

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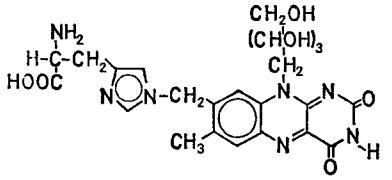
1. Succinate dehydrogenase flavocoenzyme ("SD-flavin"), previously shown to be an 8α-substituted riboflavin derivative containing a tertiary nitrogen homoconjugated to the flavin nucleus, was subjected to further hydrolysis and to reduction under acid conditions. Both conditions resulted in the liberation of 1 mole of histidine per mole of flavin. This proves histidine to be the covalent link between flavin and peptide backbone in succinate dehydrogenase and imidazole to be the tertiary nitrogen function homoconjugated to the flavin.

2. 8α-Histidyl-riboflavin has been synthesized starting from riboflavin chemically and shown to be completely identical with the natural product in optical, ESR and NMR spectra, pH-fluorescence curve and behavior on thin-layer and paper chromatography, as well as paper electrophoresis.



3. Both the natural compound isolated by acid hydrolysis of flavin peptide and the synthetic one contain two isomers, which may be separated by high voltage electrophoresis. The isomers appear to be the *N*(1)- and *N*(3)-imidazole substituted compounds. Digestion of the flavin peptide with aminopeptidase M yields only one isomer but on treatment with 6-N HCl this is gradually converted to a mixture of the two isomers. The absolute assignment of the natural isomer is suggested as 8α-[N(3)-histidyl]-riboflavin on the basis of imidazole quaternization with CH_3I, reductive cleavage of the flavin-imidazole bond and identification of the methyl-histidine liberated as 1-methyl-histidine.

In the previous paper [1] evidence was summarized that the covalently-bound flavin from the active center of succinate dehydrogenase is attached to the peptide backbone *via* the 8α-position of the flavin ring system. The nature of the substituent attached to the 8α-position was left unspecified, but the pH-fluorescence and absorption characteristics of acid-hydrolyzed SD-flavin strongly suggested that the substituent contains a tertiary nitrogen of pK_a 4.5 homoconjugated to the flavin nucleus, i.e. directly attached to C(8α).

This prediction is verified in the present paper by the demonstration that histidine is the immediate substituent and that the linkage of the peptide chain of the enzyme to the flavin is via an N—C bond from

Abbreviations and Definitions. SD-flavin, succinate dehydrogenase flavin, or 8α-[*N*(3)-histidyl]-riboflavin. For the numbering of the imidazole nucleus in histidine derivatives, *cf.* [12]. "*N*-histidyl" means (imidazole)-*N*(1) or -*N*(3), while substituents of the amino group are termed N_α. ESR, electron spin resonance; NMR, nuclear magnetic resonance.

Enzyme. Succinate dehydrogenase, or succinate : acceptor oxidoreductase (EC 1.3.49.1).

an imidazole N(3) to the 8α-methylene group of riboflavin.

In preliminary communications it has been shown that histidine is the immediate substituent on the 8α position of riboflavin [2]; histidyl-riboflavin has been synthesized and shown to be identical with the natural compound [3], and the peptide sequence around the flavin active center [4] has been determined.

EXPERIMENTAL PROCEDURE

MATERIALS AND METHODS

Benzyl-histidine, 1-methyl- and 3-methyl-histidine were products of the Cyclo Chemical Co. and aminopeptidase M of Röhm and Haas Darmstadt (Germany). All other reagents were best grade from Fluka A.G. (Buchs, Switzerland) or from Merck (Darmstadt, Germany). Tetraacetyl-riboflavin (II, R=$COCH_3$) was obtained by acetylation of riboflavin (II, R=H) [5], which in turn was a gift from Hoffmann-La Roche (Basle, Switzerland).

Melting points were determined with a Kofler block and are corrected. Thin layer chromatography was performed with plates of silica gel G (Merck). The abbreviations stand for the following solvent systems: (A) *n*-butanol—acetic acid—water (4:2:2, by vol.), (B) *n*-butanol—acetic acid—water (4:2:4, by vol.), (C) pyridine—2-N acetic acid (4:1, by vol.), (D) butanol—acetic acid—water (7:2:1, by vol.).

Light-absorption spectra were recorded on a Cary Model 14 spectrophotometer, 1 cm cells were used. ε-values are given in brackets. Nuclear magnetic resonance (NMR) studies were performed either with the 60 MHz, the 100 MHz or the 220 MHz instruments of Varian Associates using either sodium 2,2-dimethyl-2-silapentane-5-sulfonate or tetramethylsilane as reference. Chemical shift values are expressed as δ in ppm.

The Pauly reaction was performed essentially according to the published method with the modification, that the diazonium salt of sulfanilic acid was extracted into *n*-butanol [6]. This reduced the yellow background obtained otherwise on silicic acid and on paper. Quantitative Pauly reactions were measured spectrophotometrically at 520 nm in a 2-ml volume at 1-cm light path, using 5, 10 and 25-nM of histidine as a standard curve, which was found to be linear.

In the quantitative determination of amino acids the Beckman model 120 C amino-acid analyzer was used. High-voltage electrophoresis was performed on Whatman 1 or 3 papers, at 50 V/cm and 25 °C. The buffer used was 0.25-M pyridine-acetate, pH 5.5 or pH 3.4. The latter was obtained by titrating 0.5-% (v/v) pyridine to the desired pH with 5-% (v/v) acetic acid.

Liberation of histidine by acid hydrolysis from histidyl riboflavin was performed by heating 20 nM of the natural compound in 0.3 ml 6-N HCl at 125 °C for 20 h under high vacuum. The solution was evaporated to dryness at 40 °C and lyophilized for one hour. Amino-acid analysis revealed the presence of free histidine (78% yield) and traces (4 to 7%) of aspartate, glutamate, serine, and glycine which arose by breakdown of histidyl riboflavin since they were not present in the starting material. (Under these conditions riboflavin does not yield similar products). No flavin fluorescence was left, but by thin-layer chromatography (solvent A) a strong blue fluorescent spot ($R_F = 0.09$), also arising by degradation, was detected.

In the determination of pK_a values of 4 μM of the compound in 2 ml of CO_2-free water were adjusted to pH 3 with 6-N HCl and titrated under nitrogen with 0.17-N sodium hydroxide at 25 °C.

In catalytic hydrogenation 50-nM histidyl riboflavin in 1 ml trifluoro-acetic acid were maintained for 15 h at about 30 °C at 3 atm. H_2 pressure in the presence of 5 mg of 10-% Pd on charcoal, which was then filtered, extensively washed with trifluoro-acetic acid, ethanol and water. The combined washings and filtrate were concentrated to dryness. Histidine was then determined by the quantitative Pauly reaction and also identified with ninhydrin after thin-layer chromatography.

In order to obtain riboflavin from SD-flavin, the pure mixture of isomers (I_{A+B}) was reduced as stated below for V and the reaction course followed by thin-layer chromatography (cellulose, system B). The riboflavin can be isolated by extraction from dilute aqueous acid solution with *n*-butanol, while any residual SD-flavin (less than 10%) is retained in the aqueous phase.

The experiments were conducted at room temperature (23—25 °C) when not otherwise stated. All other methods were as in the previous paper [1].

SYNTHESES

8α-Bromo-tetraacetyl-riboflavin (III)

Ten g (18.4 mmol) tetraacetyl-riboflavin (II, R=$COCH_3$) in 100 ml dioxane were heated to boiling point, 80 mg dibenzoylperoxide were added and then 4.1 g (25.7 mmol) bromine in 10 ml carbon tetrachloride dropped into the solution over 5 min. After 20 min at boiling temperature the solvent was distilled off under reduced pressure, the oily residue dissolved in 100 ml chloroforme and this solution washed twice with pH-7 buffer, then with water, dried over magnesium sulfate and reduced *in vacuo* to 40 ml. To this solution were added 90 ml of dry ether, the precipitate formed was filtered and the product dried *in vacuo*: yield 10.3 g (89%). The content of 8α-bromo-tetraacetyl-riboflavin (III) was estimated from the decrease of the C(8)-CH_3 peak in the NMR sectrum (δ = 2.45 ppm) to be over 80%.

For elemental analysis the product was recrystallised from methanol/isopropanol (F = 145—150 °C). (Found: C 47.32, H 4.29, N 9.10, Br 12.96%; $C_{25}H_{27}N_4O_{10}Br$ (M_r 623.44) requires: C 48.17, H 4.37, N 8.99, Br 12.82%.) NMR (C^2HCl_3): δ = 8.09 (6-H), 7.88 (9-H), 4.72 (8-CH_2-Br) and 2.57 ppm (7-CH_3).

8α-[$N_α$-Benzoyl-histidyl(3)]-tetraacetyl-riboflavin (IV, R=$COCH_3$)

Five g (8.2 mmol) of the crude III and 4.1 g (16 mmol) $N_α$-benzoyl-histidine in 50 ml absolute dimethylformamide were heated to 80 °C for 16 h. The solvent was removed under reduced pressure, the oily residue dissolved in 100 ml chloroforme and washed three times with 50 ml 0.5-M buffer pH 7. The aqueous phase, containing the 8α-histidyl-riboflavin (IV, R=CH_3CO) was adjusted to pH 4 with glacial acetic acid, the solution saturated with ammonium sulfate and then extracted four times with *n*-butanol. The extract was dried over sodium sulfate and evaporated *in vacuo* to dryness. Traces of water were removed by azeotropic distillation with dry benzene. The residue was dissolved in 20 ml absolute methanol and absolute isopropanol added at 60 °C until the solution became turbid. After cooling overnight the precipitate was collected on a filter, and dried *in vacuo* to yield 3.3 g of the crude product (52%); F = 200—210 °C. For analysis the product was recrystallized from absolute methanol/isopropanol (F = 220—224 °C). Found: C 56.20, H 4.82, N 12.16%; $C_{38}H_{39}N_7O_{13}$ (M_r 801.79) requires: C 56.93, H 4.90, N 12.23%). λ_{max} (methanol): 445 (11700), 340 (8400) and 270 nm (34000). λ_{max} (pH 7): 448 (11700), 355 (8600) and 270 nm (32600). λ_{max} (20-% $HClO_4$): 415 sh (8700), 369 (15400) and 266 nm (33400). A_{270}/A_{448} = 2.90. NMR (10-% ^2HCl): δ = 9.12 (Im-2-H), 8.29 (6-H), 8.06 (9-H), 7.50 to 7.40 (Bz-H_5 and Im-4-H), 5.82 (8—CH_2-, 3.53 and 3.42 (His(β)-CH_2-), 2.35 ppm (7-CH_3). Under these conditions (10-% ^2HCl) the acetyl-protecting groups are hydrolysed in 10 min at room temperature.

8α-[$N_α$-Benzoyl-histidyl(3)]-riboflavin (IV, R=H)

One g (1.6 mmol) IV, R = $COCH_3$ were dissolved in 5 ml 1-N HCl and the solution allowed to stand at 24 °C for 2 h. The solvent was then reduced to 2 ml *in vacuo* and a saturated solution of sodium acetate in water slowly added until a pH of 5 was reached. The precipitate formed was collected on a filter, washed thoroughly with water, then with ethanol and dried *in vacuo* to yield 0.68 g (86%) IV, R=H. The crude compound can be reprecipitated from dilute mineral acid/sodium acetate solution to yield the pure IV (R=H), F = 209—210 °C. Found: C 56.49, H 5.06, N 14.65%; $C_{30}H_{31}N_7O_9$ (M_r 633.634) requires: C 56.87, H 4.93, N 15.47%. λ_{max} (pH 7): 448 (11800), 358 (8600) and 270 nm (33000). λ_{max} (pH 2): 448 (11400), 348 (8600) and 270 nm (33000). λ_{max} (6-N HCl): 415 sh (8800), 370 (14800) and 264 nm (32600). A_{270}/A_{448} = 2.95. NMR (10-% ^2HCl): Identical with NMR of IV, R = $COCH_3$.

8α-[Histidyl(1,3)]-riboflavin Dihydrochloride Isomer Mixture (I_{A+B})

One g (1.25 mmol) (IV) in 5 ml 6-N HCl were refluxed for 15 h. The solution was treated with charcoal, the solvent distilled off under reduced pressure and the residue dried in high vacuum. The yellow solid was dissolved in 3 ml absolute methanol and 0.5 ml of absolute methanol saturated with HCl was added. To this hot solution absolute ethanol was then added, until the solution became turbid and was left to crystallize. Several crops of crystals were collected up to a total yield of 0.6 g (82%). After recrystallization from methanol-HCl and absolute ethanol F > 350 °C decomp. Found: C 46.42, H 4.88, N 16.47, Cl 10.72%; $C_{23}H_{28}N_7O_8 \times 1.8$ HCl requires: C 46.54, H 5.01, N 15.92, Cl 10.79%. λ_{max} (pH 7): 448 (11800), 355 (8800), 269 (33000) and 221 nm (42000). λ_{max} (pH 3): 447 (11500), 345 (8900), 269 (34300) and 221 nm (43000). λ_{max} (H_0 = −3) 420 sh (8200), 372 (14400), 266 (31600), and 218 nm (37600). A_{270}/A_{448} = 2.83. NMR (10% ^2HCl): δ = 9.08 (Im-2-H), 8.40 (6-H), 8.17 (I_A, 6-H), 7.98 (I_B, 6-H), 7.71 (Im-4-H), 5.93 (8-CH_2-), 5.20 (His(α)-H and C-1′-H_2), 2.66 and 2.54 (His(β)-H_2) and 2.62 ppm (7-CH_3).

Identification of Isomers A, B. In order to elucidate the nature of the isomers, 50 mg of the mixture of I_{A+B} were dissolved in 2 ml acetic acid/acetic anhydride (1/1) containing 100 mg sodium acetate and refluxed for 15 h. The reaction course was followed by thin-layer chromatography (cellulose, system B). After removing the solvent *in vacuo*, the residue was dissolved in *n*-butanol, this solution washed with water (pH 3) and dried over magnesium sulfate. According to the methods described below for IV, R = $COCH_3$, the oily residue was subjected to methylation, reductive cleavage, hydrolysis, removal of zinc salts and in the hydrolysate a mixture of 1- and 3-methyl-histidine was demonstrated by thin-layer chromatography (cellulose, systems B, C).

Separation of Isomers I_{A+B}. During hydrolysis of IV as described above isomer A was formed first of all (*cf.* Fig. 4) while isomer B was accumulated through rearrangement of A during the last hours of the hydrolysis. Separation of the isomers was done by electrophoresis at 25 °C on Whatman No. 3 paper (46×57 cm) in pyridine-acetate buffer, pH 5.5

(3 vol. of 2-% (v/v) acetic acid: 2 vol. of 5-% (v/v) pyridine) applying 50 V/cm for 5 h. Several bands developed. The paper was dried and the strip containing the main flavin band (isomers A + B) was sewed onto another Whatman No. 3 paper and the isomers were separated by descending chromatography in *n*-butanol-acetic acid (solvent A) at room temperature for 48 h. On elution with 5-% (v/v) acetic acid 3 to 6 mg of each of the two isomers was then obtained in pure form from 30 mg mixture.

8α-[$N_α$-Benzoyl-1-methylhistidylium(3)]-tetraacetyl-riboflavin Iodide (V)

Two hundred mg (0.25 mmol) IV, R = $COCH_3$ were dissolved in 2 ml of absolute dimethylformamide and 0.5 ml methyl iodide added at 24 °C. The reaction course was followed by thin-layer chromatography (system D) and by fluorimetry: aliquot samples diluted to about 10^{-5} M in flavin were adjusted to pH 3 (citrate) and pH 7 (phosphate) respectively and the relative fluorescence intensity was measured. Quaternization of the imidazole nucleus raises the fluorescence quotient pH 3/pH 7 from 0.1 to 0.9. After completion of the reaction about 30 h a large excess (20 ml) of isopropanol was added, the precipitate collected on a filter and washed with additional isopropanol, then with ether and dried in vacuo. The crude product (160 mg, 68%) was recrystallized from methanol/isopropanol to yield the pure V, F = 183—185 °C. Found: C 50.05, H 4.81, N 10.81%; $C_{39}H_{42}N_7O_{13}I$ (M_r 943.70) requires: C 49.64, H 4.49, N 10.39%. λ_{max} (methanol): 445 (11800), 335 (8500) and 270 nm (36000). λ_{max} (pH 7): 450 (11800), 345 (8800) and 270 nm (35000). λ_{max} (6 N HCl): 415 sh (8800), 368 (15500) and 266 nm (34000). NMR (10-% ^{2}HCl): δ = 9.12 (Im-2-H), 8.26 (6-H), 8.02 (9-H), 7.49 (Bz-H_5), 7.35 (Im-4-H), 5.77 (8-CH_2-), 4.00 (Im-1-CH_3), 3.53 and 3.43 (His(β)-CH_2-) and 2.35 ppm (7-CH_3). (Under these conditions (10-% ^{2}HCl) the acetyl protecting groups are hydrolysed.)

Liberation of 1-Methylhistidine from V. Fifty mg of the pure V were dissolved in 5 ml acetic acid—trifluoroacetic acid (9:1, by vol.), the solution heated to boiling and 200 mg of zinc dust added in several portions over 30 min. Formation of tetraacetyl-riboflavin (II) was detected by diluting a sample with water, extracting the aqueous phase with chloroform and comparing the organic extract by thin-layer chromatography with authentical II. After completion of the reaction (appr. 40 min), unreacted zinc and inorganic salts were filtered off, the filtrate evaporated in vacuo and the residue refluxed 15 h in 6-N HCl. After removal of the acid in vacuo the residue was dissolved in a minimum of water and the bulk of zinc salts was separated by preparative thin-layer chromatography on cellulose (system C). The ninhydrin active band was extracted with 1-N acetic acid, the extracts reduced *in vacuo* and the residual solution compared with histidine, 1-methylhistidine and 3-methylhistidine on thin-layer chromatography (cellulose, system C and B) [7]. The eluted solution showed a single spot identical with 1-methyl-histidine both with regard to the R_F-values (*cf.* Table 1) and to the coloration of the ninhydrin reaction (histidine: grey-violet: 1-methylhistidine: green-blue 3-methylhistidine: blue-violet).

RESULTS

Demonstration of Histidine in Acid-Hydrolyzed SD-Flavin

Upon careful evaluation we found that SD-flavin obtained as described in the previous paper [1] consists, in fact of two components (I_{A+B}). Unless otherwise indicated in the discussion to follow, acid-hydrolyzed histidylriboflavin ("SD-flavin") denotes the mixture A + B as obtained on hydrolysis of the peptides at 95 °C in 6-N HCl.

On more drastic hydrolysis of this flavin mixture (125 °C, 6-N HCl) an α-amino acid is liberated in about 80% yield which has been identified as histidine by thin-layer chromatography, by amino-acid analysis and by the appearance of a positive Pauly reaction (Table 1). These hydrolytic conditions result in extensive destruction of the riboflavin moiety: presumably flavin is initially liberated as 8α-hydroxy-riboflavin [1]. This compound, however, easily undergoes "self dehydrogenation":

$$8\text{-}HOCH_2\text{-}Fl_{ox} \rightarrow 8\text{-}OCH\text{-}Fl_{red}H_2 \xrightarrow{[O]} 8\text{-}OCH\text{-}Fl_{ox} + H_2O.$$

The intermediate radical as well as the aldehyde product may then undergo various decay and self-condensation reactions.

Catalytic hydrogenation in the presence of Pd on charcoal at room temperature for 16 h in CF_3COOH with 3 atm. H_2 also results in the nearly quantitative liberation of free histidine, accompanied by irreversible reduction of the flavin in the benzenoid sub-nucleus to yield pteridine derivatives [8]. If, however, the reduction is performed in the presence of Zn, the flavin moiety is quantitatively split from the mixture A + B, yielding riboflavin as shown by chromatographic comparison with authentic material, as well as by absorption spectra and by fluorescence.

It has to be taken into consideration that reductive cleavage of the bond between flavin and histidine in SD-flavin cannot be performed in the fully-reduced or leuco-state of the flavin. Consequently, for optimal cleavage of histidylflavin conditions should be so adjusted as to leave the reversible reduction of the

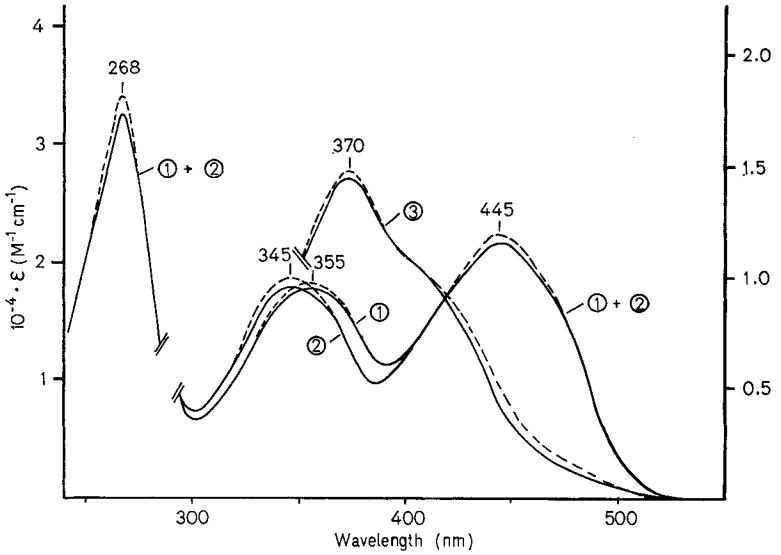


Fig. 1. *Electronic spectra of synthetic (——) and natural (– – – –) 8α-histidylriboflavin*; ① at pH 7, neutral molecule; ② at pH 3, monocation, protonated at the imidazole nucleus; ③ in 6-N HCl, trication

flavin moiety to the leuco state incomplete; *e.g.*, catalytic reduction with H_2 should be performed only in CF_3COOH [8].

The conditions required for the liberation of histidine from SD-flavin and the yields have been found to be quite comparable to those needed to liberate histidine and imidazole from the model compounds 3-benzylhistidine and 8α-(*N*-imidazolyl)-3-methyllumiflavin [1], respectively.

Synthesis and Structure of 8α-Histidylriboflavin

The demonstration of the presence of histidine in acid-hydrolyzed SD-flavin and the information presented in the preceding paper [1] permit identification of this compound as 8α-(*N*-histidyl)-riboflavin.

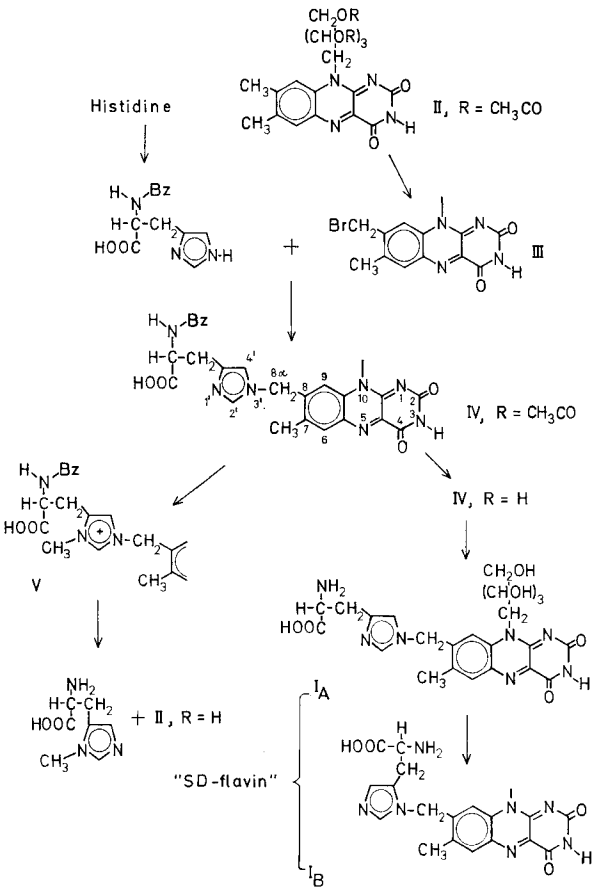
The absorption spectra of histidylriboflavin isolated from succinate dehydrogenase and of synthetic material in the neutral and protonated forms are shown in Fig. 1. The acid-hydrolyzed SD-flavin shows a pK_a of appr. 4.7 as determined by titration and fluorescence quenching (for the pK_a's of each component, *cf.* Table 1). Since we found substitution of an imidazole proton of histidine with the benzyl group to decrease the imidazole pK_a from 6.0 to 5.3, we had to expect the flavin to decrease the basicity of the imidazole even further, since the flavin has an π-electron withdrawing effect comparable with that of a *p*-nitrobenzyl residue.

Substitution of the riboflavin residue on one of the two ring nitrogens explains why the Pauly reaction is negative in both, 8α-histidylriboflavin (Table 1) and in the parent peptide, and becomes positive on drastic acid hydrolysis or reduction. Since both the carboxyl and amino groups are unsubstituted in 8α-histidylriboflavin, the compound gives a characteristic, positive ninhydrin reaction. Lastly, in accord with proposed structure and previous data for the peptide the compound migrates on high voltage electrophoresis as a cation at pH 3.4 and as a neutral compound at pH 7.

The question as to which of the two ring nitrogens of histidine is the site of attachment of the flavin is dealt with in the next part of this paper.

The synthesis of SD-flavin was carried out according to the following scheme: The most critical step was the 8α-monobromination since it could easily proceed towards the dibromo stage [4]. Alkylation of imidazole by 8α-bromoflavin (III), occurred smoothly in dimethylformamide in the absence of base. Excess base would remove an 8α-methylene proton from the product IV (Ghisla and Hemmerich, unpublished results), and the resulting carbanion would undergo decay by oxidation or disproportionation. For this reason all SD-flavin derivatives are extremely base labile.

The product IV is homogeneous, although each imidazole-N of histidine can, in principle, react. Fortunately the less hindered *N*(3)-isomer IV (as proved below) was formed in large excess and was chromatographically pure after one recrystallization. Surprisingly, the subsequent acid hydrolysis of the protecting groups proceeded with an acid catalyzed isomerization to yield the isomer mixture A + B (see below) to which the structures I_{A+B} were assigned.



Scheme 1. *SD-Flavin Synthesis and Degradation*

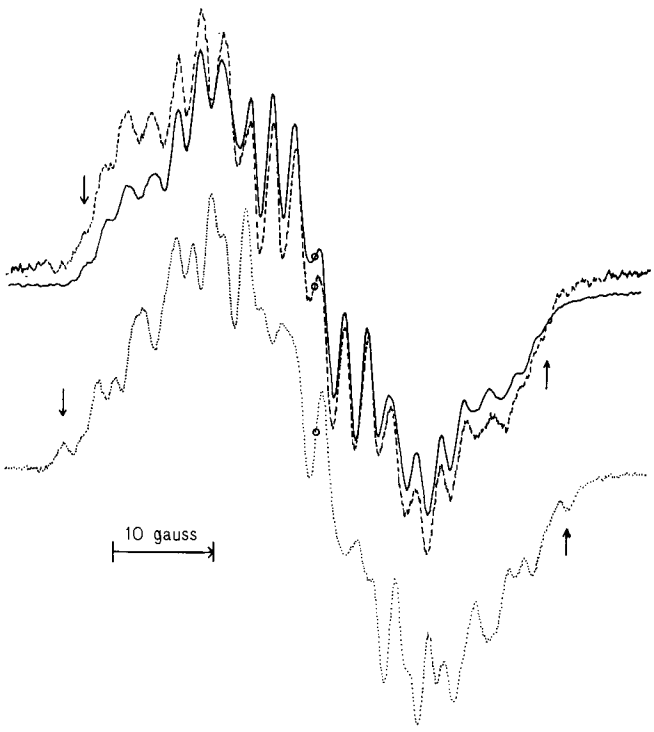


Fig. 2. *ESR hyperfine structure of synthetic (——) and natural (– – –) 8α-histidylriboflavin and of riboflavin (·····) reduced with $TiCl_3$.* Semiquinone cation radicals in 6-N HCl. The arrows indicate the outermost lines of the spectrum. Conditions: Varian E-3 spectrometer at 25 mW power in an anaerobic flat cell; modulation amplitude = 0.5 G, temperature = 25 °C. Isomers A and B exhibit identical ESR spectra

Identity of Natural and Synthetic Compounds and Assignment of Isomers

Final proof of the structure of 8α-histidylriboflavin from succinate dehydrogenase has come from the identity of the synthetic and natural compounds. Fig. 1—3 show the identity of the optical and ESR spectra and pH-fluorescence curves of natural and synthetic 8α-histidylriboflavin.

In paper and thin-layer chromatography as well as in high-voltage electrophoresis both the synthetic and natural compounds showed the presence of the same two components, which have been designated as A and B (Table 1). These components were considered to be isomers, representing substitution at N(1) and N(3) of the imidazole ring repectively. Isomerization of the natural compound occurs during hydrolysis of the peptide in 6-N HCl, for it can be avoided by using aminopeptidase M for hydrolysis of the peptide [4, 9], which yields exclusively isomer A (Table 1). The same isomer is the first product of histidine N_α-debenzoylation in the course of the synthetic procedure as demonstrated in Fig. 4 A. Isomer A rearranges on further heating to yield slowly a mixture of isomers A + B, while the hydrolysis of the benzoyl group is going to completion. The same is shown for the natural compound in Fig. 4 B. This figure also shows that isomerization A → B is unidirectional. Hence, the formation of isomer B has to be considered as an artificial acid reaction, and both isolation of the natural product and chemical synthesis yield exclusively isomer A, if acid-catalyzed isomerization is avoided.

The absolute assignment of structure to this isomer was arrived as follows: 8α(N_α-benzoylhistidyl-) tetraacetylriboflavin (IV), obtained from 8α-bromoteraacetylriboflavin (III) and N_α-benzoylhistidine was subjected to quaternization of the imidazole nucleus by means of CH_3I in dimethylformamide to give V. Proof of the quaternary structure V was obtained by nuclear magnetic resonance (*cf.* Table 1) and by the pH dependence of fluorescence (Fig. 3) reflecting loss of the quenching which was due to imidazole deprotonation. The flavin-imidazole bond was subsequently cleaved reductively by metallic Zn in acid and, after debenzoylation in 6-N HCl and removal of the major part of zinc salts by

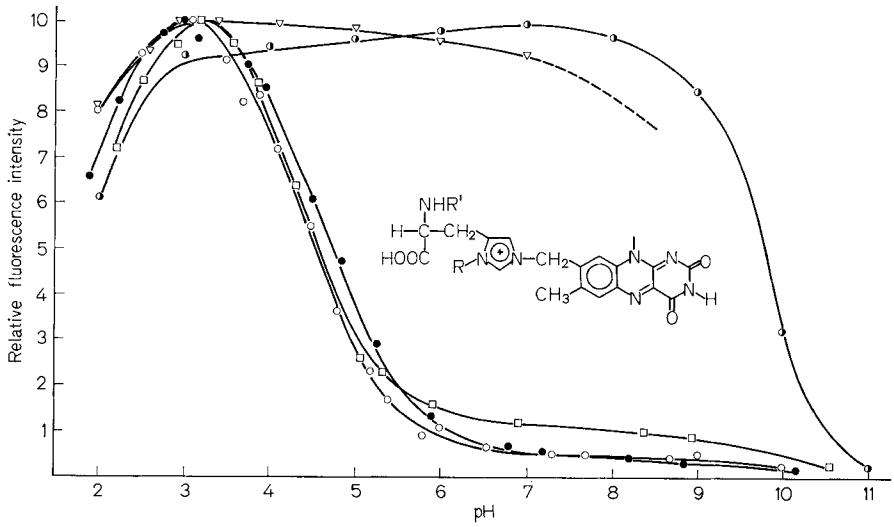


Fig. 3. *pH-dependence of fluorescence.* Natural SD-flavin A + B (□——□), synthetic isomer A (●——●), synthetic isomer B (○——○), riboflavin (◑——◑) and compound V (▽——▽)

Table 1. *Properties of 8α-histidylriboflavin isomers A + B and of methylhistidines*
TLC = thin-layer chromatography

Compound		pK_a (25 °C) From titration	pK_a (25 °C) From fluorescence	R_F values in paper chromatography (System A)	R_F values on TLC (cellulose) (System B)	Migration in TLC relative to FMN [a]	Electrophoretic mobility at pH 5.5 [b]
8α-Histidylriboflavin (natural)	(I_A)	—		0.4	0.30	0.15	−0.34
	(I_B)	—	4.6 [c]	0.5	0.35	0.18	−0.28
8α-Histidylriboflavin (synthetic)	(I_A)	4.8	4.7	0.4	0.30	0.15	−0.34
	(I_B)	4.5	4.5	0.5	0.35	0.18	−0.28
Product of aminopeptidase M digestion of flavin peptide		—	—	0.4	—	—	−0.34
1-Methylhistidine		6.2	—	1.03	0.06 [d]	0.25	−2.15
3-Methylhistidine		5.6	—	1.24	0.10 [d]	0.30	−1.82

[a] In *n*-butanol—acetic acid—water (5:3:2, by vol.), stationary phase cellulose.
[b] In arbitrary units, taking the difference in migration of FMN over that of riboflavin as +1.
[c] Mixture A + B.
[d] System C.

preparative thin-layer chromatography, the ninhydrin-positive fraction was eluted and compared with methylhistidine isomers. As demonstrated in Fig. 5, the methylhistidine obtained from SD-flavin is identical with 1-methylhistidine (and differs from 3-methylhistidine) as regards R_F and ninhydrin color. SD-flavin is represented, therefore, by structure I_A, *i.e.* 8α-(histidyl-3-) riboflavin (*cf.* Scheme 1).

NMR Spectra of Isomeric Histidylriboflavins

Assuming that the two isomeric 8α-histidylriboflavins differ only on the particular imidazole ring nitrogen at which riboflavin is substituted, it was decided to analyze the NMR spectra of the two compounds in detail in the hope that this might yield additional evidence as to which is the *N*(1)-, which the *N*(3)-substitution product. Although decisive information regarding this question has not emerged from NMR studies, other interesting differences between the isomers have been detected: The NMR spectra at 60 MHz of the two isomers, measured in the tri-cationic state, are reproduced in Fig. 6. The spectra have been analyzed by comparison with those of riboflavin, FMN, *N*(1)- and *N*(3)-methylhistidines and benzylhistidines and are in agreement

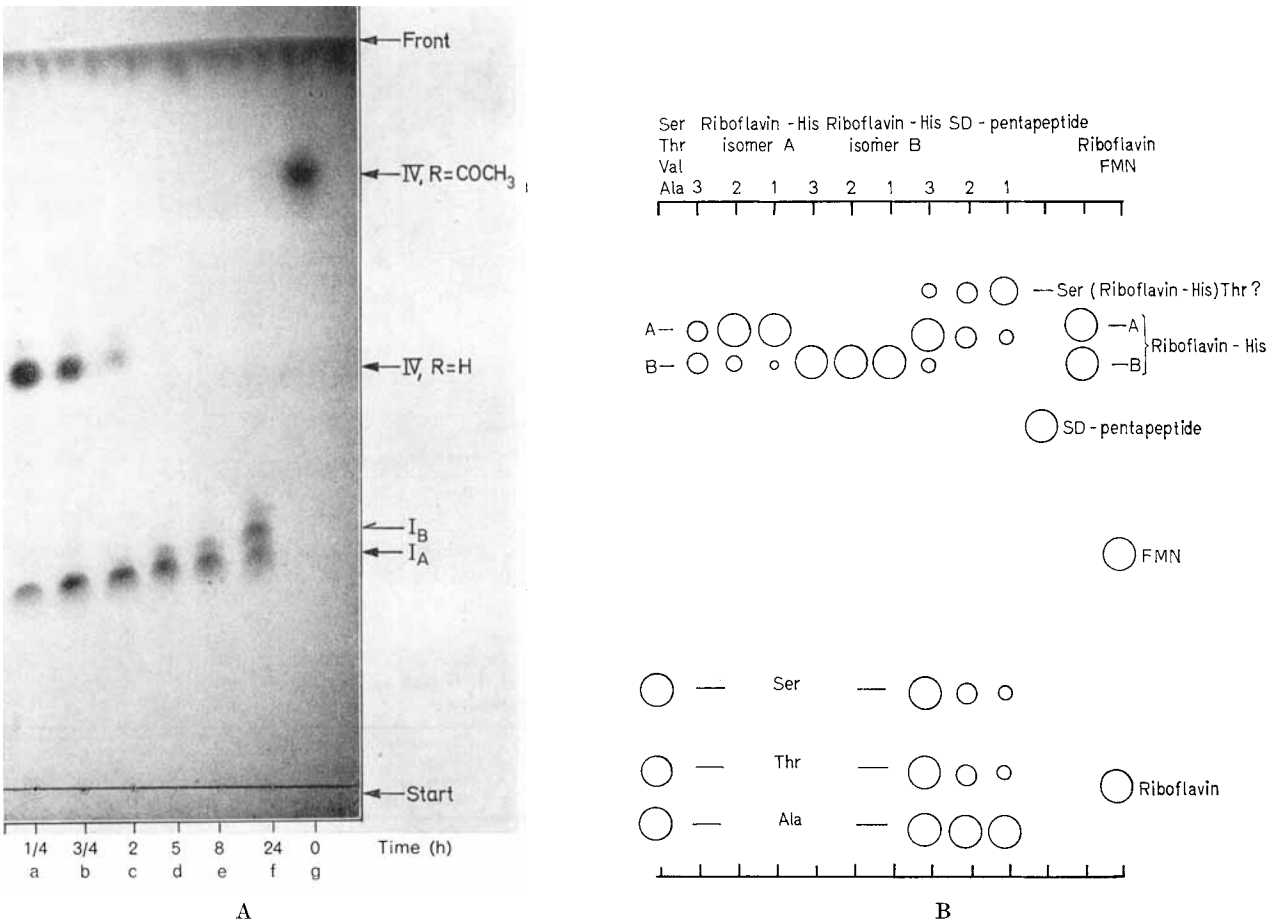


Fig. 4. (A) *Hydrolysis of the 8α-[Nα-benzoylhistidyl]-tetraacetylriboflavin* (IV, R = $COCH_3$) followed by thin-layer chromatography (system B): Zero time (g); after 15 min of refluxing in 6-N HCl the acetyl-protecting groups are quantitatively removed to give the corresponding 8α-[*N*α-benzoylhistidyl(3)]-riboflavin (IV, R = H) (a, upper spot). With increasing time this is completely hydrolysed to I_A (b, c, d, lower spots). I_A then slowly isomerises to I_B (e, f, upper spots). All spots detected by fluorescence. (B) *Course of acid hydrolysis of flavin pentapeptide and acid-catalyzed isomerization of 8α-histidylriboflavin.* Descending paper chromatography of the samples indicated: solvent system A on Whatman paper No. 1 for 15 h. Acid hydrolysis was in 6-N HCl at 95 °C for the following periods: samples number 1, 195 min; 2, 315 min; 3,13 h. All spots were ninhydrin-positive except for FMN and riboflavin on the extreme right, which are detected by fluorescence. The intensity of the ninhydrin color is denoted by the size of the spot. Note that valine runs off the paper under these conditions

with positional assignments in the literature [6, 10; also Lauterwein, Lhoste and Hemmerich, unpublished results]. The NMR-data are compiled in Table 2. The only significant NMR-difference of the isomers $I_{A,B}$ regards C(9)H of the flavin nucleus, not the imidazole CH-groups. This is easily understood for the SD-monocation, where the imidazole is protonated so that the two N-atoms become electronically more equivalent. Neutral or alkaline SD-flavin tends to undergo slowly 8α-autoxidation and disproportionation [9]. Hence, small amounts of radical impurities arise easily causing extensive NMR-line broadening. Differences between the isomers are observed particularly in the signals of the ribityl protons (in the range of 3.88 to 5.30 ppm). Although the number of ribityl protons detected is the same for A and B, their line positions are dramatically shifted. It is of interest in this context that riboflavin and FMN also differ significantly from each other in the position of the signals of the C-2, C-4, and C-5 protons of the ribityl chain. It is important to note that the 7-CH_3 group is present and intact in SD-flavin in contrast to the assumptions of others [11].

Identity and Origin of SD-Flavin Isomer B (I_B)

It has been shown above by chemical degradation that SD-flavin isomer A is identical with riboflavin

Table 2. *NMR data of 8α-substituted flavins and of imidazole-substituted histidines as δ values in ppm*
TMS = Tetramethylsilane; DSS = Sodium 2,2-dimethyl-2-silapentane-5-sulfonate

Sample	Solvent[a]	Frequency	Reference	Im(2)-H	Im(4)-H	Im-CH_3	His(β)-CH_2[b]		Fl(6)-H	Fl(9)-H	Fl(8)-CH_2-	Fl(7)-CH_3	Reference
8α-Br-Ac_4-Riboflavin (III)	C^2HCl_3	60	TMS	—	—	—	—		8.09	7.88	4.72	2.57	
Nα-Bz-Ac_4-SD-Flavin (IV)	10% $^2HCl/^2H_2O$	60	DSS	9.12	7.42	—	3.53	3.42	8.29	8.06	5.82	2.35	
Nα-Bz-1′-CH_3-Ac_4-SD Flavin (V)	10% $^2HCl/^2H_2O$	60	DSS	9.12	7.35	4.00	3.53	3.43	8.26	8.02	5.77	2.35	
SD-Flavin A (I_A)	10% $^2HCl/^2H_2O$	60	DSS	9.06	7.70	—	3.66	3.54	8.38	8.00	5.93	2.62	
	$^2H_2O/^2HCO_3^-$	220	DSS	7.78	7.10	—							c
SD-Flavin B (I_B)	10% $^2HCl/^2H_2O$	60	DSS	9.08	7.70	—	3.66	3.54	8.40	8.17	5.90	2.62	
	$^2H_2O/^2HCO_3^-$	220	DSS	7.78	7.18								c
Riboflavin (II, R = H)	10% $^2HCl/^2H_2O$	60	DSS	—	—	—	—		8.29	8.20	—	2.58	
8α-Morpholino-Lumiflavin	10% $^2HCl/^2H_2O$	60	DSS	—	—	—	—		8.78	8.50	4.80	2.80	[1]
L-3-CH_3-Histidine	10% $^2HCl/^2H_2O$	60	DSS	8.85	7.54	3.94	3.58	3.48	—	—	—	—	
L-1-CH_3-Histidine	10% $^2HCl/^2H_2O$	60	DSS	8.82	7.61	3.96	3.66	3.52	—	—	—	—	
L-$N\alpha$-Bz-3-Benzylhistidine	10% $^2HCl/^2H_2O$	60	DSS	8.84	7.29	—	3.46	3.33	—	—	5.32	—	[9]
L-$N\alpha$-Bz-3-pNO_2-Benzylhistidine	10% $^2HCl/^2H_2O$	60	DSS	9.03	7.41	—	3.61	3.48	—	—	5.61	—	[9]

[a] In 10-% ^2HCl histidine N α- and imidazole-moieties and the flavin nucleus are full protonated.
[b] As AB part of the histidine ABC-system.
[c] Flavin resonances are strong broadened due to radical formation.

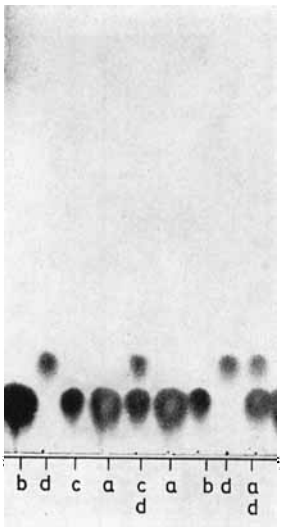


Fig. 5. *Comparison by thin-layer chromatography (cellulose, System C).* (a) the degradation product of V, (b) histidine, (c) 1-methylhistidine, (d) 3-methylhistidine. Green-blue, grey-violet, green-blue, and blue-violet colors developed in a, b, c and d respectively after ninhydrin treatment

linked via C(8α) to the imidazole N(3) of histidine. Isomer A, when subjected to hydrolytic conditions, is converted to isomer B, while th reverse reaction with pure B is not observed. Hence, formation of B is an acid catalyzed reaction and is artifactual. While it is understandable, that chemical as well as biological synthesis of SD-flavin results in attack of the flavin at the less hindered imidazole N(3), and while it is also conceivable that upon heating in acid slow equilibration between N(3) and the more hindered N(1) takes place, it remains to be explained why this reaction does not reach an equilibrium, but appears to be irreversible.

We have, therefore, subjected the A + B mixture to re-acetylation, N-quaternization and cleavage of the flavin-imidazole bond, whereafter the histidine part was found as a mixture of isomeric methylhistidines, as expected, which proves structure I_B for isomer B. Still, it remains curious that the more hindered B appears to be the more stable isomer, and we intend, therefore, to investigate the problem further by studying metal chelation reactions of A and B, where only A should allow tridentate metal/coordination at the histidine site.

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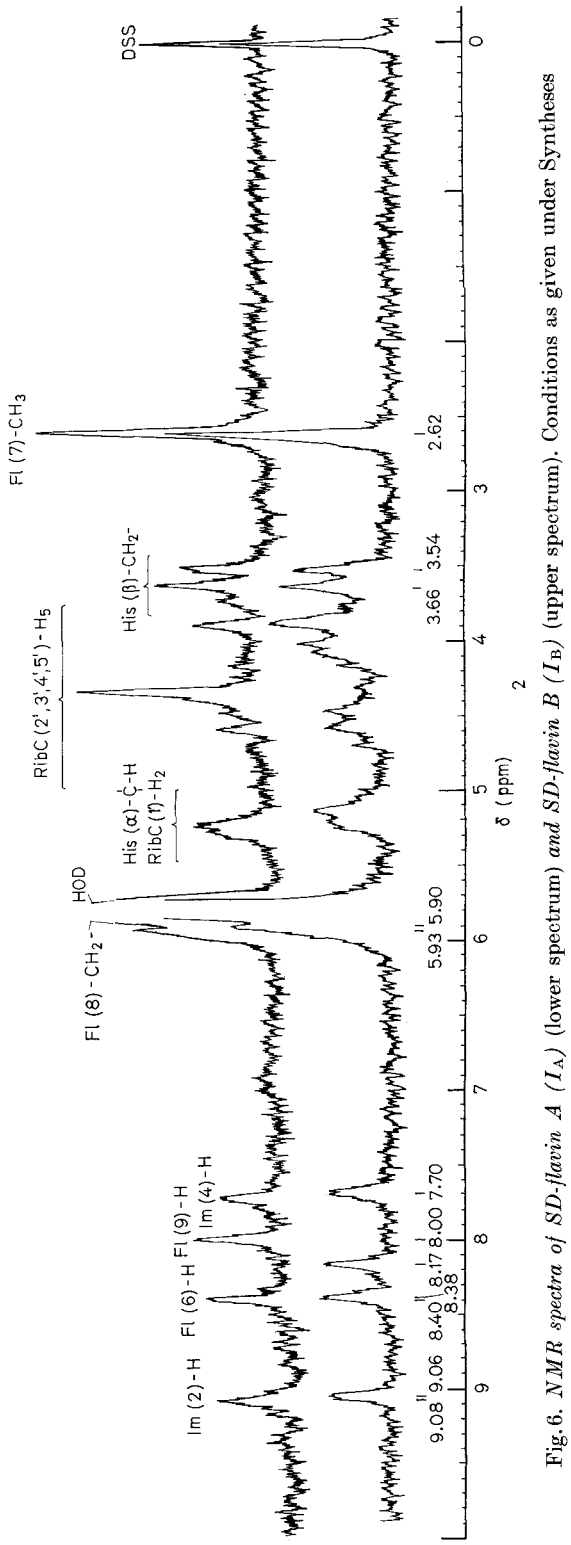

Fig. 6. *NMR spectra of SD-flavin A (I_A)* (lower spectrum) *and SD-flavin B (I_B)* (upper spectrum). Conditions as given under Syntheses

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