ON THE STRUCTURE OF SUCCINIC DEHYDROGENASE FLAVOCOENZYME

P. HEMMERICH
Fachbereich Biologie, University of Konstanz, Germany

A. EHRENBERG, W. H. WALKER, L. E. G. ERIKSSON
Medicinska Nobelinstitutet, Institutionen för Biofysik, Stockholms Universitet, Sweden

J. SALACH, P. BADER and T. P. SINGER
Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco and Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, USA

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1. Introduction

Several problems concerning the structure of the so-called covalently bound flavin remain to be resolved [1–3]. This type of flavin is mostly, if not exclusively, connected with succinate dehydrogenase flavoprotein from aerobic cells ("SD-flavin") and differs from acid-extractable flavin of the same tissue by the following main characteristics: pH-dependence of the fluorescence, optical spectra and alkaline photolysis. SD-flavin is present as "SD-FAD" in the protein [1] but yields "SD-FMN" and "SD-riboflavin" on stepwise acid and/or enzymic hydrolysis. The "SD" prefix means that the flavin is covalently substituted by a group X, which is also covalently connected to the protein backbone or, after proteolysis, to a residual peptide or a final amino acid residue.

The following main questions remain unresolved: 1. At which position of the flavin nucleus is the connection to group X? 2. What is the nature of the group X? 3. Which is the C-terminal amino acid and how is it connected to group X? The present communication deals with the first of these questions.

2. Methods

SD-flavin was isolated at a level of degradation which contains no peptide bonds but leaves the isoalloxazine nucleus and group X intact and ensures maximal flavin yield. We found an appropriate starting material in beef heart ETP_H, a particulate preparation of the inner mitochondrial membrane. An abstract of the purification has been given [2] and details will be given elsewhere [4]. The main steps consist of removal of heme and non-covalently bound flavin (step 1) by extraction with trichloroacetic acid and HCl-acetone, followed by hydrolysis with trypsin and chymotrypsin (step 2), chromatography on Florisil (step 3), and on Sephadex G 25 (step 4), total hydrolysis in 6 N HCl (12 hr, 95°) under N_2 (step 5) and final chromatography on Sephadex G 10 (step 6).

We discovered that direct HCl-treatment with omission of step 4 but inclusion of another Florisil step after step 5 resulted in liberation of riboflavin, though the removal of non-covalently bound flavin in step 1 was complete according to fluorometric analysis [2]. Further inspection revealed that SD-flavin was slowly degraded to riboflavin, perhaps on Florisil. This transformation could not be a hydrolytic process, since total hydrolysis of Kearney's [1] pure SD-flavin on the hexapeptide level did not liberate riboflavin. The different course of reaction with the crude preparations was traced to the presence of tryptophan, arising from peptide impurities, which are largely removed in step 4. The slow breakdown of SD-flavin to give riboflavin is most likely a reduction

with tryptophan as reducing agent.

Hydrolysis of purified SD-flavin peptide in the presence of tryptophan imitated the breakdown of the crude material, i.e., liberation of riboflavin could be demonstrated, but only to a minor extent. It should be noted that the pure SD-flavin hexapeptide contains no tryptophan [1].

In order to locate the point of connection between "X" and the flavin nucleus we have compared SD-flavin and normal flavin with respect to pH-dependence of fluoresence, light absorption spectra, and ESR hyperfine pattern of the radical cation. These properties will not be greatly affected by impurities from hydrolyzed protein. Further, these methods allow us to deal with the rather small amounts of SD-flavin available ($\sim 10^{-5}$ Moles).

The ESR spectra were taken in 12 or 6 N HCl. The solutions had a high viscosity due to residual amino acids. Model experiments showed that with these solvents an optimized resolution was obtained. Reduction of oxidized flavin to the radial cation was accomplished by addition of $TiCl_3$. Slight excess of paramagnetic Ti^{111} was ascertained to have no influence on the ESR spectra in the $g = 2.000$ region.

3. Results and discussion

The pH-dependence of the fluorescence of SD-flavin (fig. 1, curve 1) shows a pK in the region of 4.4 which cannot be assigned to a pK of the flavin nucleus, either in the ground or in the excited singlet state, as shown by comparison with lumiflavin (curve 2). It also cannot be due to adenine-flavin interaction, since AMP may be split from SD-flavin by mild acid hydrolysis without change of fluorescence-pH pattern [1]. The fluorescence quenching of 8α-NH_3^+-lumiflavin (curve 3) at pH ≥ 7 may be explained by deprotonation of 8α-NH_3^+. The optical spectra exhibit significant differences between SD-flavin and riboflavin (fig. 2) in both the cation and the neutral states. SD flavin cation and 8α-substituted flavin cations show the same hypsochromic shift (fig. 2). The similarity of these shifts in the case of 8α-substituted cations as different structurally as HO-CH_2- (378 nm), CH_3CONH-CH_2- (380 nm) and ^+H_3N-CH_2- (370 nm), may be due to restricted hyperconjugation at the 8α-center (fig. 6). The location of the second absorption maximum in

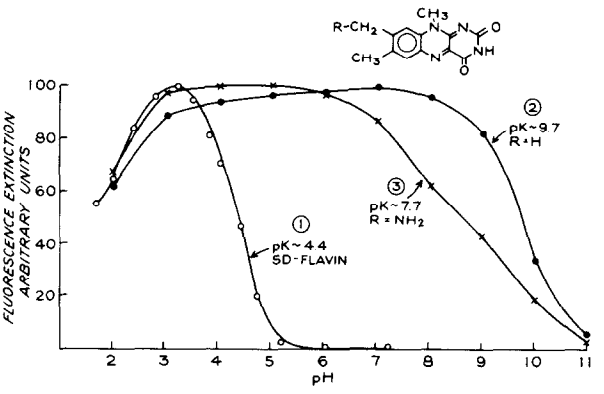

Fig. 1. pH-dependence of flavin fluorescence measured at concentrations $\sim 10^{-6}$ with a Beckman fluorometer, excitation 460 nm, emission 520 nm.

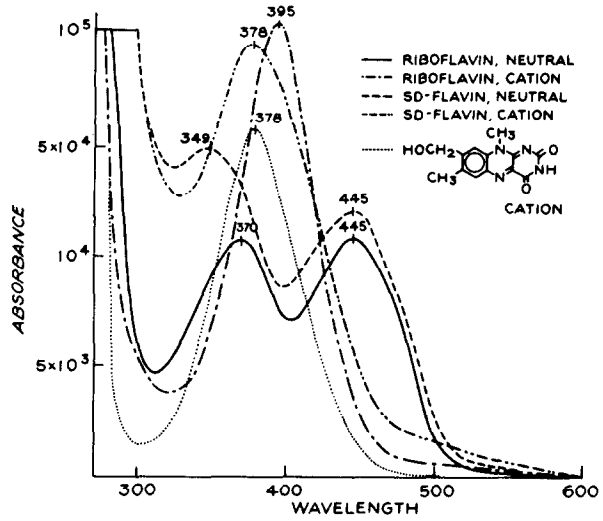

Fig. 2. Neutral and cation spectra of flavoquinone species measured with a Cary 14 spectrophotometer.

the corresponding neutral compounds is 359, 358 and 351 nm, respectively, compared to 349 nm for the pure SD-flavin and 368 nm for 8α-unsubstituted flavin.

Fig. 3 shows two typical elution patterns from Sephadex G 10 (step 6), with and without omission of step 4 (cf. above). Flavin fraction III (fig. 3) was identified as riboflavin by TLC (thin layer chromatography) in 3 different solvents, by neutral and cation light absorption spectra (λ_{max} = 370 and 395 nm, respectively (fig. 2)), fluorimetry (absence of the pH-

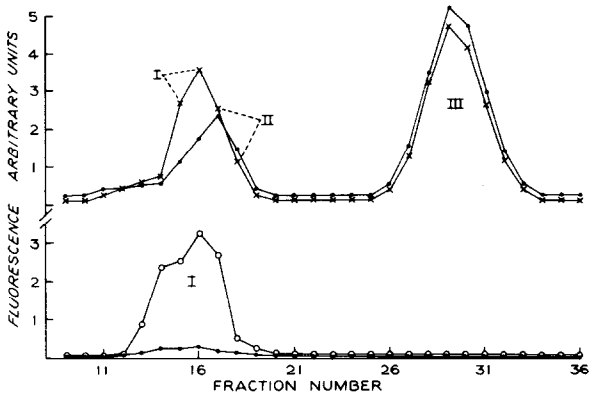

Fig. 3. Fluorescence of fractions eluted from Sephadex G 10. Upper curves, fractionation of preparations in which step 4 of the isolation procedure was omitted and Florisil chromatography after step 5 was included. Lower curves, fractionation of preparations where step 4 was included and subsequent Florisil chromatography omitted. Fluorescence measured as in fig. 1, o—o and x—x at pH 3.4, ●—● at pH 7.0.

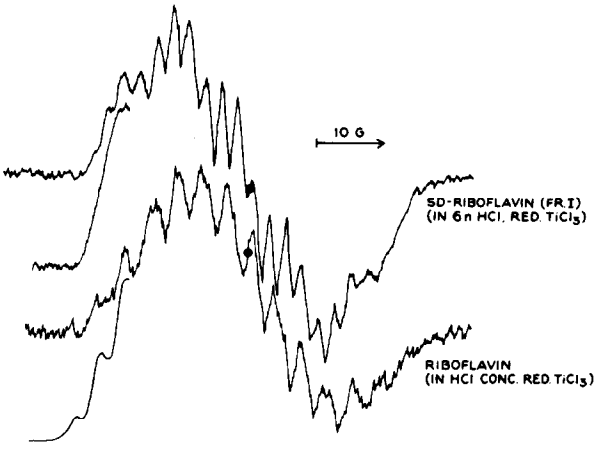

Fig. 5. ESR hyperfine structure for non-photolyzed flavin species measured as in fig. 4.

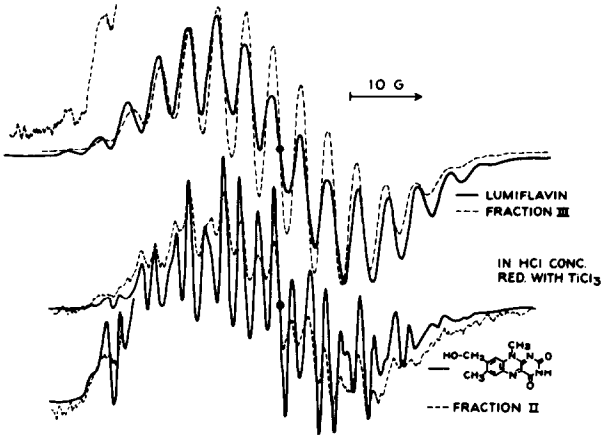

Fig. 4. Comparison of ESR hyperfine structure for photolyzed and model flavins in 6 N HCl reduced with $TiCl_3$, measured with a Varian E 3 at room temperature at 3428 ± 60 G, 10–200 mW, 9.54 GHz, modul. amplit. 0.5–1.5 Gauss.

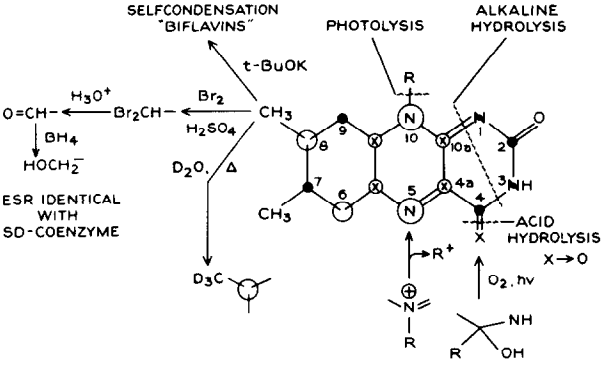

Fig. 6. Map of flavin submolecular structure and reactivity. Atoms in open circles represent points of high spin density. The spin density at atoms in crossed circles cannot be evaluated whereas spin densities at atoms marked by closed circles are low (positions 7, 9) or immeasurable small (positions 1, 2, 3, 4). The reactivities concern removal of substituents from the flavin nucleus as reviewed earlier [2] and the course of selective oxidation of 8-CH_3 [10]. The model compounds mentioned in the text were obtained in this way and their synthesis will be described in a forthcoming paper.

dependence characteristic of SD-flavin, fig. 1), and alkaline photolysis yielding lumiflavin, which was identified by the ESR hyperfine pattern of the radical cation (fig. 4).

The properties of fractions I and II on TLC, compared to fraction III, indicated an additional polar group in the molecule. Both fractions I and II still contained considerable amounts of amino acid impurities, as shown by the E_{267}/E_{445} ratio of 5.7, compared with 2.7 for pure riboflavin. The SD-flavin fluorescence was present only in fraction 1, which also showed a new ESR pattern, typical for pure, unaltered SD-riboflavin and quite different from that of riboflavin (fig. 5). Fraction II showed normal flavin fluorescence and upon alkaline hydrolysis gave a new flavin compound with an ESR hyperfine pattern very similar to that of 8α-hydroxylumiflavin (fig. 4).

It has been pointed out earlier [2,3] that the site of flavin peptide connection in SD-flavin cannot be at positions 1, 2, 3, 4, 5 and 10 of the flavin nucleus (fig. 6). Therefore only positions 6 to 9 of the flavin nucleus remain as potential binding sites. ESR data [5–7,10] indicate that model flavin radicals in all three states of protonation (anion, neutral, cation) have spin densities which are high in positions 5, 6, 8 and 10 (open circles, fig. 6), low in positions 7 and 9, and negligible in positions 1 to 4 (closed circles, fig. 6). Since SD-flavin cation radical shows an ESR hyperfine pattern significantly different from the analogous one of normal flavin, peptide connection must be located in one of positions 6 or 8. However, position 6 can be eliminated for the following reasons. It is chemically inert in all media except concentrated sulfuric acid, whereas the methyl group in position 8 undergoes a variety of reactions even under physiological conditions, e.g., deuteration [8] and self-condensation [9]. The reason for the reactivity in the 8α-position is strong hyperconjugation, comparable to the case of nitrotoluenes. Substitution in position 6 also alters the absorption spectrum drastically, whereas substitution at 8α does not [10].

If there is a hydrophilic functional group X present in 8α-position of SD-flavin, loss of two strong proton hyperfine couplings in the ESR can be expected compared to normal flavins. One ESR-active hydrogen atom is removed by group X. A second one would not show up any longer in $X\text{-}CH_2\text{-}FlH_2^+$ compared to $CH_3\text{-}FlH_2^+$ (fig. 6), since a large or hydrogen-bonded X would impede the rotation of the side chain around the CH_2-Fl bond. This loss of two ESR-active protons upon substitution of only one α-hydrogen by a large or strongly solvated functional group is a phenomenon well documented in flavin radicals [5,7].

On comparing the ESR-spectra of riboflavin and SD-riboflavin (fig. 5) one indeed observes the two main hyperfine lines and the reduction of total signal width from 52 to 46 Gauss. Furthermore, the widths of the partly resolved hyperfine lines is reduced from 3.9 for riboflavin to 2.3 G for SD-riboflavin. A similar reduction (total width 61 → 53 G, line width 4.0 → 2.5 G) is found upon comparison of lumiflavin and 8α-hydroxy-lumiflavin (fig. 4).

We conclude that position 8α is the flavin-peptide bridging site in SD-flavin. The precise nature of the group X awaits further elucidation.

Acknowledgements

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