THE COVALENTLY BOUND FLAVIN OF *VIBRIO SUCCINOGENES* SUCCINATE DEHYDROGENASE

William C. KENNEY

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94147 and Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, USA

and

Achim KRÖGER

Institut für Physiologische Chemie und Physikalische Biochemie der Universität München, 8000 München 15, Goethestrasse 33, FRG

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1. Introduction

Until recently there has been no exception to the rule that succinate dehydrogenase from aerobic cells contains FAD in covalent linkage to the polypeptide chain while in anaerobic cells the flavin is non-covalently bound [1]. In addition, the kinetic and regulatory properties of the enzyme from the latter sources are different from the enzyme from aerobic cells. In facultative anaerobes succinate dehydrogenase either has properties in between those of the aerobic and anaerobic types or two types of enzyme are present depending on the conditions of growth: a typical aerobic, mitochondrial type and a cytoplasmic one, resembling in properties the fumarate reductase of obligate anaerobes. These two types of enzymes are under separate genetic control [1]. An exception to this rule has now been found in that succinate dehydrogenase from the anaerobic bacterium *Vibrio succinogenes* contains flavin in covalent linkage to the protein. In this organism fumarate serves as the terminal electron acceptor; i.e., its reduction to succinate by succinate dehydrogenase, which functions metabolically as a fumarate reductase, is coupled to the oxidation of formate to bicarbonate, allowing the formation of one mole of ATP [2–4].

The present collaborative study originated from the observation that a membrane fraction, isolated after treatment of the bacterial cells with EDTA and lysozyme, followed by osmotic shock, contains as much as 70–80% of the total flavin in covalently bound form [3]. This report demonstrates that the covalently bound flavin is associated with succinate dehydrogenase and its structure is 8α[*N*(3)-histidyl]-FAD, the same structure as is found in mammalian succinate dehydrogenase [5].

Address correspondence to: Dr William C. Kenney, Molecular Biology Division, Veterans Administration Hospital, 4150 Clement Street, San Francisco, California 94121, USA

2. Materials and methods

The bacterium was grown and a membrane fraction isolated therefrom as previously described [4]. The lyophilized fraction, containing 130 mg protein, was suspended at 0°C in 6.7 ml H_2O and 1/10 vol. 55% (w/v) trichloroacetic acid was added. After centrifugation for 15 min at 37 000 × *g*, the resulting precipitate was successively washed with 6 N HCl/acetone, (1:120, v/v), to remove heme, 1% trichloroacetic acid acetone (1:20, v/v) then five times with 1% (w/v) trichlororacetic acid. The precipitate was suspended in 13 ml 0.1 M Tris and the pH adjusted to 8.0. The protein was digested with trypsin and chymotrypsin and the flavin peptide fraction subjected to

chromatography on Florisil [6]. The flavin fraction was taken up in water, the pH adjusted to 2, applied to a column (0.9 × 5 cm) of phosphocellulose (pyridinium form) equilibrated with 5% (v/v) formic acid, eluted with this solvent, and lyophilized.

The amino acyl flavins (riboflavin and 2′,5′-anhydro form [7]) free of all amino acids except that directly attached to the flavin, were prepared by incubation of the flavin peptide in vacuo in 6 N HCl for 15 h at 95°C, followed by high voltage electrophoresis at pH 5.0 for 2 h at 40 V/cm.

In order to ascertain the validity of the analytical procedures used for crude, membrane bound succinate dehydrogenase, parallel experiments were carried out with an inner membrane preparation (ETP) from beef heart mitochondria.

Absorption spectra were recorded with a Cary 14 spectrophotometer and corrected fluorescence spectra with a Perkin Elmer MPF-3 spectrofluorometer.

3. Results and discussion

Covalently bound flavin is the prosthetic group of the succinate dehydrogenase of *V. succinogenes*. This was shown by co-purification after solubilization from the membrane with detergents using fractional precipitation with $(NH_4)_2SO_4$ [4] and ion-exchange chromatography (fig.1). By the latter method formate dehydrogenase is separated from succinate dehydrogenase. Only in the fractions containing the latter enzyme is bound flavin found and the concentration reaches a maximum at maximal enzyme activity. The purification of activity and of the content of bound flavin is about three-fold, and the recovery is about 20%. The content of bound flavin in the peak fraction is 2.7 μmol/g protein.

Initial experiments clearly indicated that the flavin is covalently linked via the 8α position and that histidine may be the substituent involved. (See ref [9] for a discussion of properties of naturally occurring and synthetic 8α-substituted flavins.) Thus the fluorescence excitation spectrum of the crude flavin peptide showed the usual hypsochromic shift of the near ultraviolet maximum relative to riboflavin from 372–345 nm, at pH 3.4, characteristic of 8α-substituted flavins, and intense fluorescence quenching at neutral pH, suggestive of histidine substitution on the flavin.

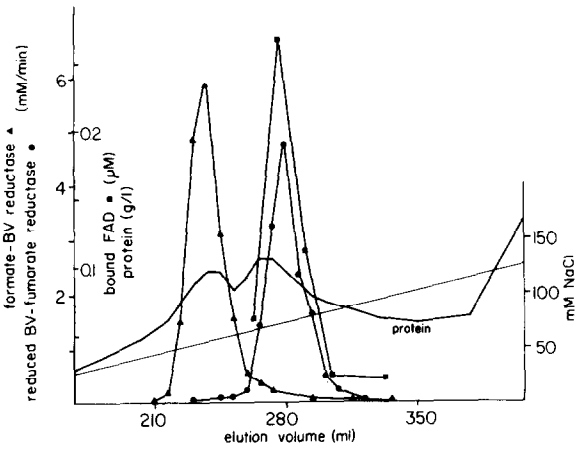

Fig.1. Chromatographic purification of succinate dehydrogenase from *V. succinogenes*. 160 mg protein of the membrane fraction [4] were extracted with 90 ml of a solution containing 1 g/l deoxycholate, 50 mM morpholinopropanesufonate, 2 mM EDTA, 1 mM NaN_3, 2 mM $Na_2S_2O_4$ and 1 mM di-isopropylfluorophosphate, pH 7.5. The mixture was stirred for 15 min at 0°C and centrifuged for 15 min at 30 000 × *g*. To the supernatant 11% polyethylene glycol-6000 were added and after 15 min the precipitate was collected by centrifugation (30 min at 30 000 × *g*). The sediment was dissolved in 6 ml 0.05% Triton X-100, 20 mM Tris–HCl, 1 mM NaN_3, 1 mM EDTA and 2 mM $Na_2S_2O_4$, pH 7.9. 33 mg of protein was layered on a DEAE–Sephadex A25 column (90 ml) which was equilibrated with the same buffer and eluted with a linear gradient of NaCl. Formate-benzylviologen (BV) reductase, reduced BV-fumarate reductase and bound flavin were measured as described earlier [4]. Protein was determined according to the Lowry et al. [8] method.

The absorption spectra of the purified flavin peptide after phosphocellulose chromatography, at two pH values, are shown in fig.2. Absorption maxima are observed at 448 nm and 351 nm, at pH 7.4 and 446 nm and 345 nm, at pH 3.6. The shift in the near ultraviolet absorption maximum as a function of pH is due to ionization of the imidazole moiety, as documented for synthetic and naturally occurring histidyl flavins [10,11].

The pK_a obtained for the fluorescence quenching process is 5.0, intermediate between that for the flavin peptide isolated from mammalian succinate dehydrogenase (pK_a 4.3) [12] which contains 8α-[*N*(3)-histidyl]-flavin and the flavin peptide of thiamine dehydrogenase (pK_a 5.8) [13] the linkage of which is via the *N*(1) position of the imidazole moiety, i.e., 8α-[*N*(1)-histidyl]-flavin. In order to

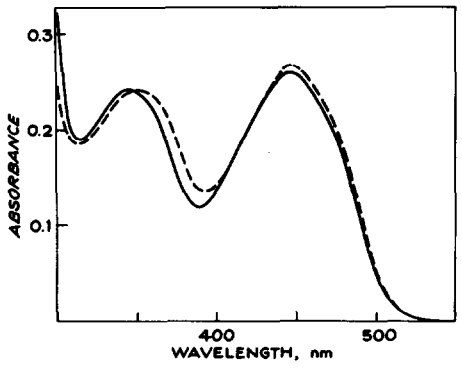

Fig.2. Absorption spectra of the tryptic–chymotryptic flavin peptide from *V. succinogenes* succinate dehydrogenase. The peptide, after the phosphocellulose purification step, was dissolved in 50 mM acetic acid (pH = 3.6) and the spectrum recorded (——). 0.3 % (by vol.) *N*-ethyl morpholine was then added (pH = 7.4) for the spectrum at neutral pH (– – –).

establish which position of the imidazole is involved in the linkage, it was necessary to characterize the pK_a of the amino acyl flavin and thus eliminate the possibility of perturbations in this value which might arise by interactions with other amino acyl residues of the peptide.

The fluorescence spectrum of the amino acyl flavin, obtained by acid hydrolysis of the flavin peptide, is given in fig.3. This compound, shows a pH dependent fluorescence yield, being maximal at pH 3–3.5 and decreasing to $< 15\%$ at pH > 7 (fig.4) with a pK_a of 4.8.

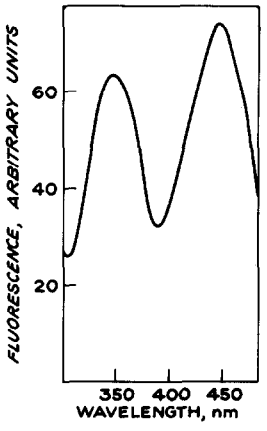

Fig.3. Corrected fluorescence excitation spectrum, at pH 3.4, of the amino acyl flavin from *V. succinogenes* succinate dehydrogenase.

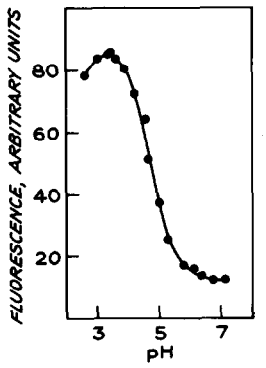

Fig.4. pH–Fluorescence profile of amino acyl flavin from *V. succinogenes* succinate dehydrogenase.

Incubation of histidyl flavins in 6 N HCl at 95°C results in formation of the 2′,5′-anhydroriboflavin derivative [10]. This was also observed in the acid treatment of the flavin peptide from *V. succinogenes* succinate dehydrogenase. The pK_a of fluorescence quenching of this derivative was also measured, as well as of the amino acyl flavins of succinate dehydrogenase isolated from a mammalian membrane preparation. These results together with mobilities on high voltage electrophoresis are summarized in table 1. The amino acyl flavins (riboflavin and 2′,5′-anhydroriboflavin forms) from *V. succinogenes* succinate dehydrogenase are identical to those obtained from mammalian succinate dehydrogenase, the structure of which is 8α-[*N*(3)-histidyl]-flavin [5]. During purification, the yields of flavin peptides and amino acyl flavins from *V. succinogenes* and mammalian succinate dehydrogenase were comparable. In the latter, the covalently bound flavin occurs as the *N*(3)-histidyl flavin derivative. No increase in fluorescence is observed at pH 7.0 on treatment of the flavin peptide from *V. succinogenes* with performic acid. An increase would be expected if cysteinyl flavins were present [14]. Thus, probably all ($> 95\%$) of the covalently bound flavin that occurs in the membrane fraction of *V. succinogenes* is *N*(3)-histidyl flavin.

Incubation of the flavin peptide with pyrophosphatase or dilute acid results in a 50% increase in fluorescence at pH 3.4, indicating the flavin to be at the dinucleotide level, presumably FAD. The pK_a of fluorescence quenching of the flavin peptide after pyrophosphatase treatment is 4.8 as is found for the

Table 1
Comparison of properties of amino acyl flavins from *V. succinogenes* succinate dehydrogenase with those of natural and synthetic 8α-histidyl flavin derivatives

Sample	Imidazole pK_a	Electrophoretic mobility pH 5.0, FAD = +1.0
Amino acyl flavins of *V. succinogenes*	4.8	−0.53
succinate dehydrogenase	4.7	−0.45
Amino acyl flavin of mammalian	4.7	−0.53
succinate dehydrogenase	4.7	−0.46
8α-[*N*(3)-Histidyl]-riboflavin	4.7[a]	−0.53
8α-[*N*(3)-Histidyl]-2′,5′-anhydroriboflavin	4.5[a]	−0.44
8α-[*N*(1)-Histidyl]-riboflavin	5.2[a]	−0.71
8α-[*N*(1)-Histidyl]-2′,5′-anhydroriboflavin	5.0[a]	−0.69

[a] From [10]

histidyl riboflavin from this enzyme (table 1). The pK_a after acid treatment, however, is 5.0. The reason for this difference is not understood, but it could arise from some hydrolysis of the peptide, e.g., cleavage at aspartyl residues, which results in slight perturbation of the acid dissociation constant of the imidazole moiety.

The results above indicate that the structure of the covalently bound flavin of *Vibrio succinogenes* succinate dehydrogenase is 8α-[*N*(3)-histidyl]-FAD (fig.5). The fact that such a structure has now been identified in an anaerobic organism further emphasizes the diversity in nature and the importance of this interesting group of vitamin B_2 derivatives.

While in this respect the dehydrogenase from *V. succinogenes* resembles the enzyme from strictly aerobic cells, in regard to catalytic properties and activation, it resembles succinate dehydrogenase from *Micrococcus lactilyticus* [15] and from other obligate anaerobes. Thus, the ratio of succinate oxidation (succinate–ferricyanide reductase [4]) to fumarate reduction (fumarate reductase [4]) is 0.21 at 25°C. This is much closer to the value of 0.03 (30°C) reported for *M. lactilyticus* than for the value characteristic of aerobic cells (62 at 38°C) [1]. The K_m values for succinate and fumarate are 8.9 mM and 0.1 mM, respectively, at 25°C, in line with its metabolic function as a fumarate reductase, similar to other succinate dehydrogenases from anaerobes [1]. Further, preliminary experiments suggest that succinate acitvates in the phenazine methosulfate assay [16] only slightly and $FMNH_2$ not at all even after preincubation with oxalacetate, whereas oxalacetate does not seem to deactivate in a manner characteristic of mammalian succinate dehydrogenase [17]. Thus, while in regard to the nature of its flavin component the enzyme from *V. succinogenes* resembles mitochondrial succinate dehydrogenase from aerobes, in its functional properties it is strictly like the enzyme from other anaerobic cells.

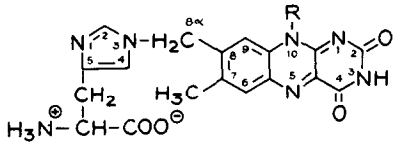

Fig.5. Structure of the covalently bound flavin from *V. succinogenes* succinate dehydrogenase. *R* is rest of FAD.

Acknowledgements

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