Identification of the covalently bound flavins of D-gluconate dehydrogenases from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* and of 2-keto-D-gluconate dehydrogenase from *Gluconobacter melanogenus*

William McINTIRE,* Thomas P. SINGER,*†§ Minoru AMEYAMA,‡ Osao ADACHI,‡ Kazunobu MATSUSHITA‡ and Emiko SHINAGAWA‡

*Molecular Biology Division, Veterans Administration Medical Center, San Francisco, CA 94121, U.S.A., †Department of Biochemistry and Biophysics and ‡Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, U.S.A., and ‡Department of Agricultural Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan

An improved method is presented for the purification of 8α -(N^1 -histidyl)riboflavin, 8α -(N^3 -histidyl)riboflavin and their 2',5'-anhydro forms, which permits the isolation of sizeable quantities of each of these compounds from a synthetic mixture in pure form. Flavin peptides were isolated from the D-gluconate dehydrogenases of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* and from the 2-keto-D-gluconate dehydrogenase of *Gluconobacter melanogenus*. After conversion into the aminoacyl-riboflavin, the flavin in all three enzymes was identified as 8α -(N^3 -histidyl)riboflavin. By sequential treatment with nucleotide pyrophosphatase and alkaline phosphatase, the flavin in each enzyme was shown to be in the dinucleotide form.

INTRODUCTION

Since the structure of the first covalently bound flavin, the prosthetic group of succinate dehydrogenase, was elucidated 15 years ago (Walker & Singer, 1970), five different types of covalently bound flavin have been identified in over 25 enzymes. Despite extensive studies in many laboratories, no relation has been found between the catalytic function of an enzyme and whether or not it will contain covalently bound flavin and, if so, what type. Thus, although the flavin components of some enzymes that function in non-phosphorylating carbohydrate metabolism in micro-organisms have been identified (Kenney *et al.*, 1979; Harada *et al.*, 1979), when several membrane-bound bacterial enzymes involved in gluconate metabolism were isolated and shown to contain covalently bound flavin (Matsushita *et al.*, 1979a, 1982; Shinagawa *et al.*, 1981) it was necessary to characterize their prosthetic groups.

The enzymes investigated in the present study, D-gluconate dehydrogenase from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* and 2-keto-D-gluconate dehydrogenase from *Gluconobacter melanogenus*, are of interest to us because of their unusual properties. All three are flavocytochromes and contain per mol 1 mol each of flavoprotein, a *c*-type cytochrome and a colourless low- M_r subunit of unknown function, which seems to be an essential constituent (Matsushita *et al.*, 1979a; Shinagawa *et al.*, 1981). The two gluconate dehydrogenases are also unusual because their cytochrome subunit is reported to contain two haem groups (Matsushita *et al.*, 1982). This raises the interesting question whether electron transport from the flavin to ubiquinone (Matsushita *et al.*, 1979b) involves both haem groups and, if so, whether they act in tandem or sequentially. As a first step in defining the function of the component subunits of

these enzymes, a collaboration between the laboratories in San Francisco and Yamaguchi was undertaken to characterize the covalently bound flavins. The present paper is an account of this work.

EXPERIMENTAL

Materials

D-Gluconate dehydrogenase from *Ps. aeruginosa* and from *Ps. fluorescens*, and 2-keto-D-gluconate dehydrogenase from *Gluconobacter melanogenus* were purified as previously described (Matsushita *et al.*, 1979a, 1982; Shinagawa *et al.*, 1981). The pure proteins were precipitated with $(\text{NH}_4)_2\text{SO}_4$ in the presence of cholic acid, centrifuged, dried *in vacuo* and stored at -20°C .

The phosphocellulose was obtained from the Gallard-Schlesinger Chemical Mfg. Corp., analytical cellulose thin-layer plates (F1440) were from Schleicher and Schuell, and preparative cellulose Uniplates (250 μm thick) were from Analtech. The ampholytes, pH 5–8, and the Sephadex G-200 (superfine grade) were from Pharmacia Fine Chemicals. Octadecylsilane-modified silica gel (flash-chromatography grade, 40 μm particle size) was manufactured by the J. T. Baker Chemical Co. The analytical octadecylsilane-bonded silica-gel (ODS-Spherogel, 3 μm particle size) column (4.6 mm $\times$ 75 mm) was from Beckman Instruments. Crystallized bovine trypsin and chymotrypsin were purchased from the Worthington Biochemical Corp., and *Escherichia coli* alkaline phosphatase and *Crotalus atrox* nucleotide pyrophosphatase were from Sigma Chemical Co.

Synthesis and purification of histidyl-flavins

A mixture of 8α -(N^1 -histidyl)-, 8α -(N^3 -histidyl)-, 2',5'-anhydro- 8α -(N^1 -histidyl)- and 2',5'-anhydro- 8α -

§ To whom correspondence should be addressed.

(*N*³-histidyl)-riboflavins was synthesized as previously described (Walker *et al.*, 1972). A solution of the crude mixture (1.6 mmol of flavin) in 8% (v/v) formic acid was applied to a 2.5 cm × 20 cm phosphocellulose column, equilibrated with the same solution. Neutral flavins were washed off the column with the same formic acid solution, whereas the charged material remained bound. The column was washed with 5% (v/v) acetic acid, and flavins held on the column were eluted with 5% (v/v) pyridine and freeze-dried. A small amount of this material was purified by paper electrophoresis at pH 5.0, with 5% (v/v) pyridinium acetate. The mobilities relative to FMN were used to identify the various histidyl-flavins (Edmondson *et al.*, 1976) and were used as standard throughout the remaining experiments. The individual histidyl-flavins were also subjected to h.p.l.c. analysis (3 µm-particle-size ODS-Spherogel column, 4.6 mm × 75 mm). The flow rate was 0.7 ml/min and the solvent 5 mM-ammonium acetate (pH 4.8)/methanol (21:4, v/v). Elution of flavins was monitored at 438 nm. The same h.p.l.c. system was used in subsequent steps to monitor flavins.

The bulk of the material eluted from the phosphocellulose column was freeze-dried and then dissolved in a minimal volume of 1% (v/v) acetic acid, and applied to a second phosphocellulose column (2.5 cm × 20 cm) equilibrated with the same solvent. This was washed with 300 ml of 1% acetic acid; then a linear gradient was applied, with 750 ml of 1% acetic acid in the mixer and 750 ml of 1% (v/v) pyridine in the reservoir, 9.25 ml fractions being collected. Histidyl-flavins were eluted in the pH range 5.9–6.8 in two peaks. Peak I (fractions 115–124) contained mostly 2',5'-anhydro-8α-(*N*³-histidyl)riboflavin and peak II (fractions 125–135) mostly 8α-(*N*³-histidyl)riboflavin, although each of these fractions contained significant amounts of the corresponding *N*¹-histidyl isomer. The material collected in peak I and that in peak II were freeze-dried separately.

The flavin from peak I was dissolved in 4 ml of 1:20-diluted Pharmalyte 5–8, mixed with a thick slurry of Sephadex G-200 (superfine grade) gel and applied in a 0.8 cm × 20 cm trough in a bed of swollen Sephadex. The bed (21.5 cm × 21.5 cm × 0.5 cm) consisted of 8 g of washed Sephadex G-200 (superfine grade) preswollen in 250 ml of water and 12.5 ml of Pharmalyte 5–8 (Frey & Rudola, 1982). (A detailed description of the preparation of the Sephadex and conditions for running the isoelectric focusing can be found in a pamphlet entitled 'Isoelectric Focusing, Principals and Methods' available from Pharmacia Fine Chemicals.) An electric field (25 W) was applied and the flavins were focused according to their pI values. The pH values along the gradient were determined by scooping out a small amount of the Sephadex, suspending it in water, centrifuging, and measuring the pH. Four major bands developed and were separately processed. The material in peak II was treated identically and the same four bands appeared on electrofocusing.

Identical bands originating from peaks I and II were combined, and the flavin in each of the four bands was eluted with 5% (v/v) acetic acid, filtered through a sintered-glass funnel and freeze-dried. Each of the resulting four samples was dissolved in a minimum volume of 5 mM-ammonium acetate, pH 4.8. The final step in purification was chromatography of each sample on a 1.5 cm × 50 cm column of 40 µm-particle-size

octadecylsilane-bonded silica-gel particles, with 5 mM-ammonium acetate (pH 4.8)/methanol (89:11, v/v) as the eluent and a flow rate of 4 ml/min. Monitoring absorbance at both 254 and 438 nm indicated that the ampholytes were eluted in the first 25 min and the flavins at about 50 min. Comparison of the absorption spectra of each flavin with published data (McIntire *et al.*, 1981) and analytical h.p.l.c. indicated that all four histidylriboflavins were pure.

Purification of flavin peptides

As a result of the isolation procedure (Matsushita *et al.*, 1979a, 1982; Shinagawa *et al.*, 1981), the enzyme preparations contained unknown amounts of poly(ethylene glycol), cholic acid and Triton X-100. It was therefore necessary to remove these agents as much as possible before proteolytic digestion. Samples of D-gluconate dehydrogenase from *Ps. aeruginosa* (25 nmol) and from *Ps. fluorescens* (22 nmol) and of 2-keto-D-gluconate dehydrogenase (16 nmol) were suspended in 0.5 ml of water, and each was twice extracted with chloroform/methanol (2:1, v/v). To each residue 0.55 ml of 5.5% (w/v) trichloroacetic acid was added. The suspensions were centrifuged, each residue was suspended in 5 ml of 1 M-Tris base and re-centrifuged, each pellet was again suspended in 0.5 ml of 75 mM-Tris/HCl buffer, pH 7.6, and 5 mg each of trypsin and chymotrypsin were added, along with a drop of toluene to retard bacterial growth.

Each of the samples was incubated for 18 h, at which time 3 mg more of each proteolytic enzyme was added and digestion continued for another 24 h. This long digestion was required because of the unusual resistance of these membrane enzymes to proteolysis. The turbid solutions were centrifuged and the reddish pellets discarded. To the supernatant solutions 50 µl of 55% (w/v) trichloroacetic acid was added and centrifugation repeated. The resulting clear solutions were applied to 0.5 cm × 5 cm Florisil columns, equilibrated with 5% (v/v) acetic acid, washed with the same solution until the absorbance at 280 nm fell below 0.02, then washed with water, and finally eluted with 20% (v/v) pyridine. To remove Florisil carried over, each eluate was freeze-dried, resuspended in a small volume of 20% pyridine and centrifuged, and the pellets were washed twice with minimal volumes of 20% pyridine. The combined clear liquids were then freeze-dried.

For optimal separation of the FAD-containing peptides from *Ps. aeruginosa* and *G. melanogenus* by t.l.c., they were converted into the riboflavin level by successive treatments with 2 µl of a 1 mg/ml solution of nucleotide pyrophosphatase in 50 mM-*N*-ethylmorpholinium acetate buffer, pH 7.6, containing 15 mM-MgCl₂ for 5 min at 37 °C, then with 2 µl of a 1 mg/ml solution of alkaline phosphatase in 50 mM-*N*-ethylmorpholinium acetate, pH 8.0, for 5 min.

The final step in purification of the flavin peptides from each dehydrogenase involved chromatography on 250 µm-thick cellulose thin-layer plates (2.5 cm × 20 cm). Solvent system C (see below) was used for the peptides from *Ps. aeruginosa* and *G. melanogenus* and solvent system D for the *Ps. fluorescens* peptide. The major yellow and fluorescent band originating from each enzyme was scraped off. These bands were, in each case, well separated from the haem and most of the ninhydrin-positive bands. The cellulose was suspended in acetone, transferred to a small column, washed with acetone, and

Table 1. Chromatographic properties and isoelectric-focusing characteristics of histidyl-flavins

The pK_a values given refer to the imidazole nitrogen, as determined by the dependence of fluorescence on pH (Edmondson *et al.*, 1976). The R_F values in the last column were determined in solvent system C.

8 α -Histidyl-flavin	pI	pK_a	Retention time in h.p.l.c. (min)		R_F
			Solvent A	Solvent B	
N^3 -	6.64	5.2	2.53	2.84	0.25
2',5'-anhydro- N^3 -	6.46	5.0	2.70	3.00	0.28
N^1 -	6.28	4.7	4.29	4.56	0.30
2',5'-anhydro- N^1 -	6.00	4.5	4.35	4.42	0.32

Fig. 1. Dependence of the fluorescence of the aminoacyl-flavin from D-gluconate dehydrogenase on pH

The aminoacyl-flavin was isolated from the dehydrogenase of *Ps. aeruginosa*, as described, and dephosphorylated to the riboflavin level. The flavin was dissolved in 3 ml of 50 mM-sodium acetate. The solution was titrated first with small portions of 5% (v/v) acetic acid, then with 100% acetic acid. After each addition the pH and the fluorescence were measured. The ● symbols represent experimental points, corrected for volume change, and the continuous line is the computer fit to the Henderson-Hasselbalch equation.

eluted with 20% (v/v) pyridine. The resulting peptides were then freeze-dried.

Generation of the aminoacyl-flavins

The flavin peptide sample was placed in a hydrolysis tube, and 0.2 ml of water and 0.2 ml of 12 M-HCl were added. The tubes were evacuated, sealed, and incubated at 95 °C for 16 h. These conditions are not harsh enough to cause significant destruction of histidyl-flavins (Edmondson *et al.*, 1976). The samples were freeze-dried and used without further purification.

Other methods

The fluorescence measurements were carried out in a Hitachi-Perkin-Elmer MPF-3 spectrofluorometer, and h.p.l.c. separations with an instrument manufactured by Altex Scientific. Effluents from h.p.l.c. were monitored

either by absorbance or by fluorescence. In the latter case a Gilson Spectra/Glo fluorimeter, equipped with a 330–400 nm-excitation filter and a 460–600 nm-emission filter, was used. For isoelectric focusing the Pharmacia flat-bed apparatus (FBE 3000) was employed. Spectra were taken on a Cary 14 spectrophotometer interfaced to a Nova 2/4 computer. The pH-fluorescence titration data were fitted by computer to the Henderson-Hasselbalch equation by using a program that we have described (Kenney & McIntire, 1983). The solvent systems used for h.p.l.c. were: A, 5 mM-ammonium acetate (pH 4.8)/methanol (21:4, v/v); B, 5 mM-ammonium acetate (pH 4.8)/methanol (3:1, v/v). The flow rate was 0.7 ml/min and the column a Beckman 3 μ m-particle-size ODS-Spherogel. Solvent systems for t.l.c. were: C, pyridine/butan-1-ol/acetic acid/water (15:10:3:12, by vol.); D, butan-1-ol/acetic acid/water (12:3:5, by vol.). Analytical-grade cellulose was the supporting phase in each case.

RESULTS AND DISCUSSION

Improved separation of synthetic histidylriboflavins

Pilot experiments on a flavin peptide isolated from the D-gluconate dehydrogenase of *Ps. fluorescens* indicated that it is likely to contain a histidylriboflavin derivative, as judged from the pH-dependence of its fluorescence. Identification of the particular isomer required the synthesis of pure 8 α -(N^1 -histidyl)riboflavin and 8 α -(N^3 -histidyl)riboflavin. Although their synthesis and separation from each other, as well as from their anhydro forms produced in the synthesis, has been described previously (Edmondson *et al.*, 1976), the separation methods used in these earlier studies were limited to processing 2–5 μ mol of material because of the limited capacity of the paper-electrophoretic and paper-chromatographic methods used. Another disadvantage of these earlier methods was that the filter paper used as well as the kerosene coolant frequently introduced impurities, removal of which necessitated additional purification steps.

In searching for alternative separation methods we found chromatography in phosphocellulose columns in conjunction with isoelectric focusing ideal for the purpose. These techniques permitted processing 20–40 μ mol of histidyl-flavin at a time and resulted in complete separation of the four forms. A final reverse-phase h.p.l.c. step also had a high capacity for these compounds and resulted in complete removal of all detectable impurities. The four pure histidyl-flavins were then identified by comparison with standards prepared by the earlier procedure (Edmondson *et al.*, 1976) from their respective electrophoretic mobilities.

Properties of histidyl-flavins

With substantial quantities of both isomers of histidylriboflavin and of their 2',5'-anhydro forms available, we determined their pI values, retention times on a reverse-phase h.p.l.c. column, and R_F values in the t.l.c. systems in current use (Table 1). Assuming that the pK_a values of the carboxy and α -amino groups in the four compounds are the same, their pI values should increase with the pK_a of the imidazole nitrogen. As seen in the Table, this proved to be the case. Significant differences in retention time in h.p.l.c. are also documented, whereas

Table 2. Characteristics of the aminoacyl-flavins isolated from various dehydrogenases

The R_F values in the last column were determined in solvent system C. Abbreviation: N.D., not determined

Source of aminoacyl-flavin	pK_a	Retention time in h.p.l.c. (min)		R_F
		Solvent A	Solvent B	
D-Gluconate dehydrogenase (<i>Ps. aeruginosa</i>)	4.74 ± 0.01	N.D.	4.54	N.D.
D-Gluconate dehydrogenase (<i>Ps. fluorescens</i>)	4.65 ± 0.02	4.31	N.D.	0.30–0.32
2-Keto-D-gluconate dehydrogenase (<i>G. melanogenus</i>)	4.68 ± 0.02	4.27	N.D.	0.30–0.32

Table 3. R_F values of flavin-containing peptides at the FAD, FMN and riboflavin level

Source of peptide	R_F value		
	As isolated	After pyrophosphatase	After pyrophosphatase and phosphatase
D-Gluconate dehydrogenase (<i>Ps. aeruginosa</i>)	0.12*	0.29*	0.13*
D-Gluconate dehydrogenase (<i>Ps. fluorescens</i>)	0.43†	0.61†	0.74†
2-Keto-D-gluconate dehydrogenase (<i>G. melanogenus</i>)	0.46*	0.68*	0.67*
	0.14†	0.24†	0.16†

* In solvent D.

† In solvent C.

the R_F values of isomeric flavins in the t.l.c. system used are not markedly different.

Identification of the aminoacyl-flavins

At the outset of this study low yields in the proteolytic digestion step and resulting limitation of material available for analysis posed a problem that hampered positive identification of the type of flavin present in the three enzymes investigated here. This was traced to the hydrophobicity of these membrane-bound proteins (Matsushita *et al.*, 1979a, 1982; Shinagawa *et al.*, 1981). The procedure was revised, as described in the Experimental section, to overcome this problem, giving satisfactory yields, which were more than sufficient for unambiguous identification of the flavin.

The dependence of fluorescence on pH of the aminoacyl-flavins from all three dehydrogenases left no doubt that an 8 α -histidyl-flavin was present. A typical titration is shown in Fig. 1. Table 2 summarizes the pK_a values and chromatographic behaviour of all three aminoacyl-flavins isolated at the riboflavin level. Comparison with the values for authentic samples in Table 1 proves beyond doubt that the compound isolated from all three dehydrogenases is 8 α -(N³-histidyl)riboflavin.

It remained to determine whether the flavin was present in these enzymes at the dinucleotide or mononucleotide level. To this end, the major flavin peptide from each enzyme was analysed by t.l.c. before and after treatment with nucleotide pyrophosphatase, which converts FAD-containing peptides into the corresponding FMN-containing peptides, and again after incubation with alkaline phosphatase to hydrolyse the

5'-phosphate group. The results, presented in Table 3, clearly show that the flavin is at the FAD level in all three dehydrogenases.

This investigation was supported by the Program Project HL16251 from the National Institutes of Health, by Grant PCM19609 from the National Science Foundation, and by the Veterans Administration.

REFERENCES

- Edmondson, D. E., Kenney, W. C. & Singer, T. P. (1976) *Biochemistry* **15**, 2937–2945
- Frey, M. D. & Rudola, B. J. (1982) *Electrophoresis* **3**, 216–225
- Harada, Y., Shimizu, M., Murakawa, S. & Takahashi, T. (1979) *Agric. Biol. Chem.* **43**, 2635–2636
- Kenney, W. C. & McIntire, W. (1983) *Biochemistry* **22**, 3858–3868
- Kenney, W. C., Edmondson, D. E., Singer, T. P., Nishikimi, M., Nogudri, E. & Yagi, K. (1979) *FEBS Lett.* **97**, 40–46
- Matsushita, K., Shinagawa, E., Adachi, O. & Ameyama, M. (1979a) *J. Biochem. (Tokyo)* **85**, 1173–1181
- Matsushita, K., Shinagawa, E., Adachi, O. & Ameyama, M. (1979b) *J. Biochem. (Tokyo)* **86**, 249–256
- Matsushita, K., Shinagawa, E. & Ameyama, M. (1982) *Methods Enzymol.* **89**, 187–193
- McIntire, W., Edmondson, D. E., Hopper, D. J. & Singer, T. P. (1981) *Biochemistry* **20**, 3068–3075
- Shinagawa, E., Matsushita, K., Adachi, O. & Ameyama, M. (1981) *Agric. Biol. Chem.* **45**, 1079–1085
- Walker, W. H. & Singer, T. P. (1970) *J. Biol. Chem.* **245**, 4224–4225
- Walker, W. H., Singer, T. P., Ghisla, S. & Hemmerich, P. (1972) *Eur. J. Biochem.* **26**, 279–289