IDENTIFICATION OF THE COVALENTLY-BOUND FLAVIN OF L-GALACTONOLACTONE OXIDASE FROM YEAST

William C. KENNEY, Dale E. EDMONDSON, Thomas P. SINGER, Morimitsu NISHIKIMI+, Etsuko NOGUCHI* and Kunio YAGI*

Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143 and *Molecular Biology Division, Veterans Administration Hospital, San Francisco, CA 94121, USA,* *+Department of Medical Chemistry, Kochi Medical School, Nangoku-shi 781–51* and **Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan*

Received 5 October 1978

1. Introduction

Until recently 11 enzymes were known to contain covalently-bound flavin, linked by way of the 8α or the C(6) position of the flavin ring to a histidine or cysteine residue in the enzymes [1,2]. A covalently bound flavin in an additional enzyme, L-galactonolactone oxidase from yeast has been shown [3]. This enzyme is considered to catalyze the last step of L-ascorbic acid biosynthesis in this organism. An isofunctional enzyme present in animal tissues, L-gulonolactone oxidase had been reported [4] to contain a flavin linked to the N(1) position of histidine. Accordingly, it is phylogenetically interesting to identify the structure of the flavin of the yeast enzyme. The present study revealed that yeast L-galactonolactone oxidase also contains 8α-[N(1)-*histidyl*]FAD.

2. Materials and methods

L-Galactonolactone oxidase was purified from baker's yeast as in [3]. A flavin peptide was obtained from the purified L-galactonolactone oxidase as follows. About 14 mg protein containing 25 nmol flavin were precipitated with 5% (w/v) trichloroacetic acid at 0°C and the suspension was stirred for 15 min and centrifuged at 12 000 × *g* for 10 min. The supernatant and 'oil' phase (which was formed as a result of the presence of the detergent, Tween 20) were carefully removed and the precipitate was suspended in acetone and centrifuged as above, followed by a further wash with 1% (w/v) trichloroacetic acid. The precipitate was suspended in 1.5 ml 0.1 M *N*-ethylmorpholinium acetate (pH 8.5) and 0.07 mg each trypsin and chymotrypsin/mg protein were added. After incubation for 4 h at 38°C, the incubation mixture was centrifuged to remove turbid material and lyophilized. The sample thus obtained was re-lyophilized from 5% (v/v) acetic acid, dissolved in this solution, and applied to a column (0.5 × 5.5 cm) of phosphocellulose (pyridinium form, equilibrated with 5% (v/v) acetic acid). The column was washed with 5% acetic acid, then with 1% acetic acid, and the flavin band was eluted with a buffer containing 0.02 M pyridine–0.2 M acetic acid (pH 3.7).

The aminoacyl flavin was obtained from the flavin peptide by incubation of the latter in 6 N HCl at 92°C for 16 h in vacuo, followed by high-voltage electrophoresis at pH 5.0 or pH 6.2 [5]. Incubation with pyrophosphatase, BH_4^- reduction, and performic acid oxidation were as in [6]. Corrected fluorescence excitation spectra were measured using a Perkin Elmer MPF-3 spectrofluorometer.

3. Results and discussion

As shown in table 1, the fluorescence of the flavin peptide isolated from L-galactonolactone oxidase is

Table 1
Fluorescence properties of flavin peptide from L-galactonolactone oxidase

Treatment of peptide	Fluorescence measurements pH	Relative fluorescence[a] (%)
None	7 0	4
None	3 4	43
Pyrophosphatase	3.4	73
Pyrophosphatase, then BH_4^-	3 4	3
Pyrophosphatase, then oxidation at 0°C	3.4	100
Pyrophosphatase, oxidation at 38°C	3.4	100

[a] Relative to fluorescence after pyrophosphatase treatment and performic acid oxidation

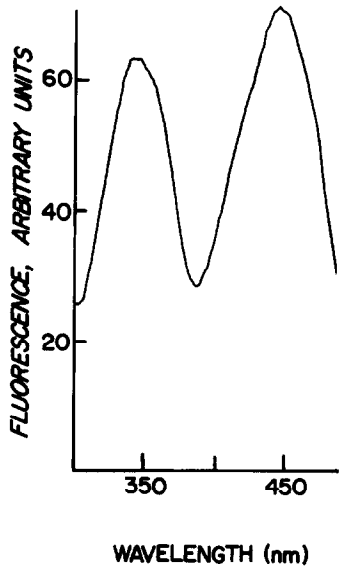

Fig 1 Fluorescence excitation spectrum of tryptic–chymotryptic flavin peptide (FMN level) of L-galactonolactone oxidase, at pH 3.25.

greatly quenched at neutral pH as compared with pH 3 4, indicating that the substituent in the flavin is a histidyl residue, which ionizes between these pH values. The fluorescence at pH 3.4 also increases on nucleotide pyrophosphatase treatment, suggesting that the flavin is at the dinucleotide level. The fluorescence excitation spectrum of the flavin peptide at pH 3.25 following hydrolysis to the mononucleotide level, is reproduced in fig.1. The hypsochromic shift of the second excitation maximum from 372 nm (in FMN) to 346 nm is compatible with substitution at 8α.

These data leave two possibilities for the structure of the flavin in L-galactonolactone oxidase 8α-[N(1)-*histidyl*]FAD and 8α-[N(3)-*histidyl*]FAD. The fact that BH_4^- reduces the flavin, as judged by the fluorescence quenching (table 1), indicates that the N(1) isomer is present since this reacts with BH_4^-, while the N(3) isomer does not [5,6]. In accord with this, the pK_a of fluorescence quenching of the tryptic–chymotryptic peptide was 5.6 both before (fig.2A) and after (fig.2B) oxidation with performic acid, very nearly the same value as reported for similar peptides from L-gulono-γ-lactone oxidase [4], thiamine dehydrogenase [7], and β-cyclopiazonate oxidocyclase [8]. In contrast, the pK_a values of flavin peptides containing 8α-[N(3)-*histidyl*]FAD are nearly 1 pH unit lower [4–8]. Following acid hydrolysis to the anhydro form of the aminoacyl flavin, the pK_a observed (fig.2C) (5.0) is as expected for 8α-[N(1)-

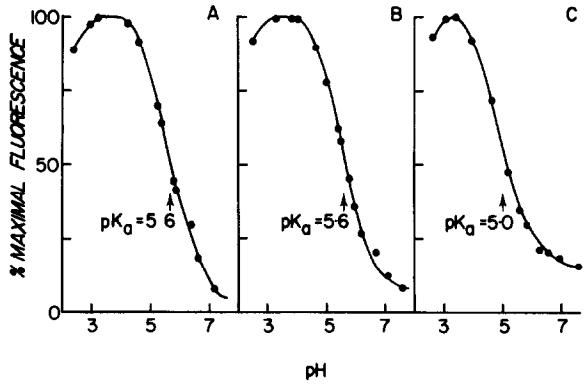

Fig.2A pH Dependence of fluorescence intensity of tryptic–chymotryptic flavin peptide The flavin peptide (FMN level) was dissolved in 1 mM citrate–phosphate buffer and the pH adjusted by μl additions of 3 N HCl or 3 N NaOH

Fig.2B pH Dependence of fluorescence intensity of tryptic–chymotryptic flavin peptide oxidized with performic acid Conditions were as in fig.2A.

Fig.2C pH Dependence of fluorescence intensity of aminoacyl flavin Conditions were as in fig.2A

Table 2
Mobility of aminoacyl flavin in high-voltage electrophoresis

Sample	Migration	Mobility relative to FMN
FMN	12.5	1 0
N(1)-histidylriboflavin	−10 1	−0 81
N(3)-histidylriboflavin	− 7 5	−0 60
Aminoacyl flavin	−10 1	−0 81

The flavin peptide was digested for 16 h at 92°C with 6 N HCl, then taken to dryness High-voltage electrophoresis was at pH 5 for 108 min in the system and under the conditions in [5]

histidyl]2′,5′-anhydroriboflavin [9].

Additional confirmation for the N(1) linkage was obtained by comparison of the mobilities of the product obtained on hydrolysis with 6 N HCl with those of authentic N(1) and N(3) histidylriboflavins (table 2). On high voltage electrophoresis at pH 5 the material comigrated with the N(1) isomer. Thus, the covalently bound flavin of L-galactonolactone oxidase is 8α-[N(1)-*histidyl*]FAD (fig.3).

An additional point of interest is that oxidation of the tryptic–chymotryptic flavin peptide (FMN level) with performic acid at either 0°C at 38°C increases its fluorescence at pH 3.4. This fluorescence enhancement probably indicates the presence of a tryptophan residue in the peptide which interacts with the flavin, partially quenching its fluorescence [6].

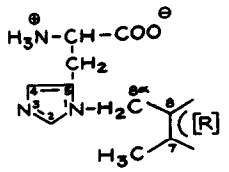

Fig.3. Structure of covalently-bound flavin of L-galactonolactone oxidase R is the rest of FAD

It is interesting from the phylogenetic standpoint that the flavin of L-galactonolactone oxidase is 8α-[N(1)-*histidyl*]FAD like that of rat L-gulonolactone oxidase. Both enzymes participate in the last step of L-ascorbic acid biosynthesis and show the similar substrate specificity. It has been conjectured [3] that the animal enzyme and the yeast enzyme evolved from a common ancestor enzyme. The present finding provides an additional piece of evidence for this hypothesis

Acknowledgements

This study was supported by the National Institutes of Health (HL-16251) and the National Science Foundation (PCM 76–03367).

References

[1] Edmondson, D E and Singer, T P (1976) FEBS Lett 64, 255–265
[2] Steenkamp, D J , McIntire, W and Kenney, W C (1978) J. Biol. Chem. 253, 2818–2824.
[3] Nishikimi, M., Noguchi, E and Yagi, K (1979) Arch Biochem. Biophys in press
[4] Kenney, W C , Edmondson, D E., Singer, T P , Nakagawa, H , Asano, A and Sato, R (1976) Biochem. Biophys. Res Commun 71, 1194–1200
[5] Kenney, W C., Edmondson, D E and Seng, R (1976) J. Biol. Chem. 251, 5386–5390.
[6] Kenney, W C , Edmondson, D E , Singer, T P , Steenkamp, D. J and Schabort, J (1976) Biochemistry 15, 4931–4935
[7] Kenney, W C., Edmondson, D E and Singer, T P (1974) Biochem. Biophys Res Commun 57, 106–111
[8] Kenney, W C., Edmondson, D. E , Singer, T P , Steenkamp, D J and Schabort, J (1974) FEBS Lett 41, 111–114
[9] Edmondson, D E. (1977) Biochemistry 16, 4308–4311