IDENTIFICATION OF THE COVALENTLY BOUND FLAVIN OF L-GULONO-γ-LACTONE OXIDASE

William C. Kenney, Dale E. Edmondson, and Thomas P. Singer

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 and Molecular Biology Division, Veterans Administration Hospital, 4150 Clement Street, San Francisco, California 94121

Hidehiko Nakagawa*, Akira Asano, and Ryo Sato

Institute for Protein Research, Osaka University, Suita, Osaka, Japan

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Summary: It has been previously reported (Nakagawa et al. (1975) J. Biochem. (Tokyo) 77, 221-232) that L-gulono-γ-lactone oxidase contains a flavin moiety covalently linked to the protein. Properties of a partially purified flavin peptide, liberated by pronase digestion, indicate the presence of histidyl flavin, but not 8α-[N(3)-histidyl]-riboflavin. In the present study, a purified tryptic-chymotryptic flavin peptide was acid-hydrolyzed to the amino acyl flavin (a mixture of its riboflavin level as well as its 2',5'-anhydro form). High voltage electrophoresis, fluorescence excitation spectrum, and pK_a of fluorescence quenching, in comparison with synthetic compounds all show that 8α-[N(1)-histidyl]-riboflavin is the structure of the covalently bound flavin of this enzyme.

In enzymes containing a covalently bound flavin prosthetic group, the amino acid substituent on the flavin has been found to be either a histidyl or a cysteinyl residue while the site on the flavin moiety is, in each case, at the 8α position. (For a review see refs. 1-3). So far, four types of covalently linked flavins have been characterized: 8α-[N(3)-histidyl]-FAD, as in succinate dehydrogenase (4,5)› D-6-hydroxynicotine oxidase (6), and bacterial sarcosine dehydrogenase (7); 8α-S-cysteinyl-FAD, as in monoamine oxidase (8); 8α-S-cysteinyl-8α-hydroxy-FAD,as in Chromatium cytochrome $\underline{c}_{552}$ (9);and 8α-[N(1)-histidyl]-FAD (10), as in thiamine dehydrogenase (11) and β-cyclopiazonate oxidocyclase (12).

In a recent report (13) a flavin peptide was isolated from L-gulono-γ-lactone oxidase, an enzyme which catalyzes the terminal reaction of the microsomal ascor-

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* Present address: Department of Biochemistry, University of Southern California, Los Angeles, California 90033.

bate synthesizing system in mammalian liver except that of primates and guinea pig. The isolated flavin peptide from pronase digests of the enzyme showed: a) a hypsochromic shift of the absorption peak in the near ultraviolet region; b) a pH-dependent fluorescence yield, with a maximum at pH 4.0 and minimal fluorescence at pH > 7; and, c) a pK_a of 5.8 for this fluorescence quenching process. These results suggested the flavin to be linked through the 8α position to a histidyl residue although the properties did not agree with those of a flavin linked to the N(3) position of histidine. Similar pK_a values for the fluorescence quenching have been noted for thiamine dehydrogenase (11) and β-cyclopiazonate oxidocyclase (12) which have recently been shown to contain histidyl flavin linked via the N(1) position (10). As shown in the present paper the prosthetic group of L-gulono-γ-lactone oxidase also contains 8α-[N(1)-histidyl]-riboflavin.

MATERIALS AND METHODS

L-gulono-γ-lactone oxidase was purified through the first DEAE-Sephadex A-50 chromatography as previously reported (13). The N(1) and N(3) isomers of 8α-histidylriboflavin and their acid-modified forms were synthesized and purified as described respectively by Edmondson *et al.* (10) and by Walker *et al.* (5).

The protein was prepared for proteolytic digestion essentially as described (13), except that the incubation in trichloroacetic acid was omitted. The acid-precipitated pellet in 0.1 *M* Tris, pH 8.0 was digested with trypsin and chymotrypsin (0.1 mg each/mg protein) for 4 hr at 38°. After cooling to 0°, 0.1 volume 55% (w/v) trichloroacetic acid was added, and the soluble flavin peptide was removed from acid-insoluble material by centrifugation. The supernatant from trichloroacetic acid (5%) precipitation (∿ 60 nmol of flavin) was applied at 4° to a 0.9 x 6 cm column of Florisil previously equilibrated with 5% (v/v) acetic acid, and the flavin peptide was eluted as before (13). After lyophilizaton and redissolving in a minimum volume of 5% (v/v) formic acid, it was chromatographed on a column (0.9 x 6 cm) of phosphocellulose (pyridinium form) equilibrated with the same solvent. The flavin peptide, slightly retarded in this column, was eluted with 5% formic acid, and lyophilized.

The amino acyl flavin was prepared by acid hydrolysis of the flavin peptide in 6 N HCl at 95° for 16 hrs *in vacuo*. After solvent removal, the amino acyl flavin was purified by high voltage electrophoresis at pH 6.25, 50 V/cm, 2 hrs. Corrected fluorescence excitation spectra were measured using a Perkin Elmer MPF-3 spectrofluorometer.

RESULTS AND DISCUSSION

The value of the pK_a of fluorescence quenching of the pronase peptide reported by Nakagawa *et al.* (13) (pK_a = 5.8) and the fact that the absorbance of the peptide was bleached by BH_4^-, an unusual property of flavin, suggested that the flavin might be linked to the N(1) of the imidazole because of similar prior observations on tryptic-chymotryptic peptides of thiamine dehydrogenase and β-cyclopiazonate oxidocyclase which have recently been shown to contain 8α-[N(1)-histidyl]-riboflavin (10). Because of the limited quantity of flavin peptide available (cf. MATERIALS AND METHODS) it was decided to obtain proof for this assignment by acid hydrolysis of a partially purified tryptic-chymotryptic flavin peptide instead of enzymatic hydrolysis to the amino acyl flavin which would require considerably more material.

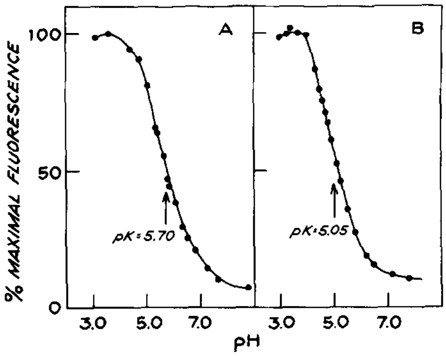

Fig. 1. A. pH Dependence of fluorescence yield of tryptic-chymotryptic flavin peptide. Flavin solutions were dissolved in 1 mM citrate-phosphate buffer and the pH adjusted by additions of microliter quantities of 3 N HCl or 3 N NaOH. B. pH-Dependence of fluorescence yield of amino acyl flavin. The conditions were the same as in A.

The properties of the tryptic-chymotryptic flavin peptide used in the present study were very similar to those of the pronase peptide (13). The fluorescence of the tryptic-chymotryptic flavin peptide (after nucleotide pyrophosphatase treatment) is markedly pH dependent (Fig. 1A) with a pK_a of 5.7 which is in good agreement with the pK_a reported for the pronase peptide (13). At pH 3.4, the corrected excitation spectrum of this flavin peptide (FMN level) has maxima at 346 and 447 nm (Fig. 2). The hypsochromic shift of the near UV maximum relative to FMN is indicative of substitution at the 8α position of the flavin moiety (1). These results thus confirm attachment of a histidyl substituent to the 8α position.

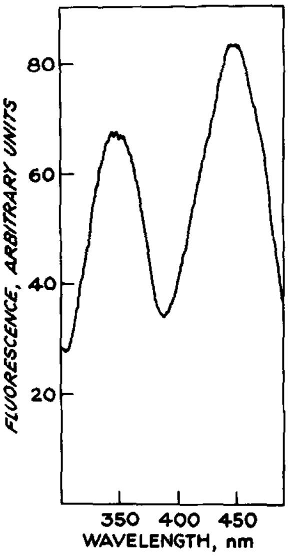

Fig. 2. Fluorescence excitation spectrum of tryptic-chymotryptic flavin peptide (FMN level) of L-gulono-γ-lactone oxidase, pH = 3.4.

Acid hydrolysis of the flavin peptide under conditions expected to cleave all of the amino acids except that directly attached to the flavin (6 N HCl, 95°, 16 hrs) results in an amino acyl flavin that is resolved into two components on high voltage electrophoresis at pH 6.25. As shown in Table I, one component (∿ 14% of the total fraction) co-migrates with 8α-[N(1)-histidyl]-riboflavin while the other component (86% of the total fraction) co-migrates with acid-modified 8α[N(1)-histidyl]-riboflavin. Acid treatment has been shown to modify 8α-[N(1)-histidyl]-riboflavin, 8α-[N(3)-histidyl]-riboflavin, as well as riboflavin, in the ribityl side chain to form the 2',5'-anhydroriboflavin analog (D.E. Edmondson, manuscript in preparation) and the anhydro derivative is the predominant form when the flavin is phosphorylated. Treatment of the acid-modified amino acyl flavin with $NaBH_4$ results in reduction of the flavin to a form which is non-fluorescent. Similar observations have been reported for the pronase flavin peptide (13) and is an empirical test for distinguishing the N(1) and N(3) histidyl flavin isomers (10).

The pH-fluorescence profile of the acid-modified amino acyl flavin shows a

TABLE I

IDENTIFICATION OF THE AMINO ACYL FLAVIN OF L-GULONO-γ-LACTONE OXIDASE

Flavin analog	Electrophoretic mobility to anode, pH 6.25 (cm)	Reduction by $NaBH_4$	pK_a of fluorescence quenching
8α-[N(3)-Histidyl]-riboflavin	1.25	No	4.7
8α-[N(3)-Histidyl]-2',5'-anhydroriboflavin	1.20	No	4.5
8α-[N(1)-Histidyl]-riboflavin	2.90	Yes	5.2
8α-[N(1)-Histidyl]-2',5'-anhydroriboflavin	2.20	Yes	5.0
Amino acyl flavins from L-gulono-γ-lactone oxidase	2.85	-	5.1
	2.10	Yes	5.0

pK_a for fluorescence quenching of 5.05 (Fig. 1B) which is identical with that of synthetic 8α-[N(1)-histidyl]-2',5'-anhydroriboflavin (10) but differs from 8α-N(3)-histidyl]-riboflavin or its 2',5'-anhydro form by 0.3 - 0.5 pK units, respectively (5). For the amino acyl flavin component co-migrating with 8α-[N(1)-histidyl]-riboflavin, a pK_a value of 5.1-5.15 was estimated. This value is very close to the value of 5.20 determined for synthetic 8α-[N(1)-histidyl]-riboflavin (10).

These results, summarized in Table I, demonstrate that the identity of the covalently bound flavin of L-gulono-γ-lactone oxidase is 8α-[N(1)-histidyl]flavin (Fig. 3).

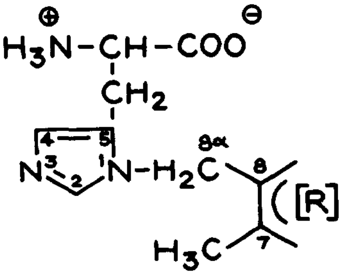

Fig. 3. Structure of the histidyl flavin of L-gulono-γ-lactone oxidase. R is rest of flavin molecule.

Preliminary evidence suggests the flavin to be at the dinucleotide level since incubation with 0.1 M HCl, 38°, 16 hr, results in an increase of the fluorescence of the flavin peptide by 42% at pH 3.4. Similarly, incubation with pyrophosphatase results in a 26% increase in fluorescence.

Although this increase is less than that observed for pyrophosphatase cleavage of FAD, or of the FAD peptide from thiamine dehydrogenase (11), it is quite possible that the prolonged contact with acid preceeding the proteolytic digestion could have resulted in some hydrolysis of the pyrophosphatase bond.

The detection of 8α-[N(1)-histidyl]-flavin in enzymes from bacteria (thiamine dehydrogenase), from mold (β-cyclopiazonate oxidocyclase) and now from mammalian origin demonstrate the diversity of this isomer of 8α-histidylflavin in nature as has been found in the case of the N(3) isomer. The reasons for the occurrence of these two isomeric forms of 8α-histidyl flavins remains, to date, unknown.

ACKNOWLEDGEMENTS

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