STRUCTURE OF THE COVALENTLY BOUND FLAVIN OF MONOAMINE OXIDASE

Edna B. Kearney, J.I. Salach, W.H. Walker, R. Seng

and T.P. Singer

Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121 and Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco, California 94122

Received December 21, 1970

SUMMARY. Flavin peptides derived from monoamine oxidase, free from succinate dehydrogenase flavin, were obtained by digestion of outer membranes of beef liver mitochondria with trypsin and chymotrypsin and purification by various chromatographic methods. The flavin peptides show the same hypsochromic shift of the optical absorption spectrum as flavin peptides from succinate dehydrogenase: the 370 mμ band of the neutral oxidized flavin is shifted to 340 mμ, whereas the cation shows a peak at 370 mμ. The ESR spectrum of the monoamine oxidase flavin cation radical also resembles that of succinate dehydrogenase flavin in that the total width is reduced from 49 G (in riboflavin) to at least 45 G, and the line width from 3.8 G (in riboflavin) to 2.3 G. The covalently bound flavins of monoamine oxidase and succinate dehydrogenase differ, however, in that the former shows the same fluorescence intensity between pH 3.4 and 8, while the latter is quenched with a pK of 4.5 ± 0.1. These observations indicate that the FAD of monoamine oxidase is covalently linked to the peptide chain through the 8α-CH_3 group of riboflavin but histidine is not the immediate substituent, as in succinate dehydrogenase. Hydrolysis of flavin peptides from monoamine oxidase in 6 N HCl at 95° yields a derivative chromatographically distinct from free flavins which is ninhydrin-positive and thus contains an amino acid bound to the 8α-position.

INTRODUCTION

Recent studies have elucidated the structure of the covalently-bound FAD at the active center of succinate dehydrogenase (SD) as a substitution of the peptide chain in the 8α-position of riboflavin (1-3) via an imidazole nitrogen of histidine (4). Histidyl-8α-riboflavin has been synthesized (5,6) and the peptide sequence around the flavin determined (7).

Although SD appears to be the only source of covalently-bound flavin in some cell types (e.g., heart, aerobic yeast (8)), there have been several reports in the literature that monoamine oxidase from liver and kidney mitochondria contains riboflavin in covalently-bound form (9-12). The evidence pre-

sented for the presence of tightly bound flavin in this enzyme (10-12) has been substantially the same as in early studies on SD (13): the flavin was not released in free form by any combination of denaturation methods but was solubilized on proteolytic digestion as a flavin peptide, whose concentration was then determined spectrophotometrically or fluorometrically. Unfortunately, published purification procedures for monoamine oxidase are such that they would tend to inactivate but not necessarily remove SD, so that the possibility cannot be ruled out that the properties so far reported for monoamine oxidase flavin are in part due to the presence of flavin originating from SD. SD contamination could not be ruled out from studies of homogeneity either, for the enzyme appears to have been isolated in several polymeric forms (11, 12), with different specific activities, so that the purity of any grown preparation is open to question.

In order to circumvent these difficulties we have elaborated a procedure for the large scale preparation of outer membranes of beef liver mitochondria virtually free from SD (an inner membrane enzyme). Flavin peptides isolated from this source material permitted the unambiguous demonstration that the prosthetic group of monoamine oxidase is FAD covalently linked to the peptide chain via the 8α-position but, unlike in SD, not through an imidazole nitrogen. Future papers will describe the purification of the enzyme from this source by a relatively simple procedure.

MATERIALS AND METHODS

Outer membrane preparations were isolated by an adaptation of the procedure of Sottocasa et al. (14). About 15 g of washed fresh liver mitochondria, suspended in 0.01 M Tris-P_i, pH 7.4, at 20 mg/ml were allowed to swell for 10 min at 0°; 0.33 vol. of 1.8 M sucrose-2 mM ATP-2 mM Mg SO_4 were added and after 10 min the suspension was gently sonicated. The sucrose concentration was brought to 0.75 M and the protein to 10 mg/ml and the suspension was centrifuged for 20 min at 34,700 x g. The supernatant, stored overnight at 0°, was recentrifuged for 30 min at 44,000 x g. The supernatant, diluted with 1

vol. of 10 mM Tris - P_i, was centrifuged for 30 min at top speed in the Spinco No. 50.1 rotor. The resulting pellet contained about 40% of the monoamine oxidase of the mitochondria 5 to 6 fold purified and 2 to 3% of the SD. Flavin peptides were isolated by a procedure to be detailed in a later publication, involving the following steps: heat and trichloroacetic acid treatment to remove free flavins, $CHCl_3$-methanol (2:1, v/v) extraction of lipids and acid-acetone treatment, trypsin + chymotrypsin digestion, chromatography of the liberated flavin peptides on Florisil, Sephadex G-25, phosphocellulose, and DEAE-cellulose, and further purification by thin layer and paper chromatography.

Monoamine oxidase activity was determined with benzylamine as substrate (15). Spectrophotometric, fluorometric, and ESR methods were as in previous work (1,2).

RESULTS AND DISCUSSION

At the outset of this work it was ascertained that preparation of monoamine oxidase, membrane-bound or extracted, which were essentially free from SD, contained substantial quantities of flavin which was not released in free

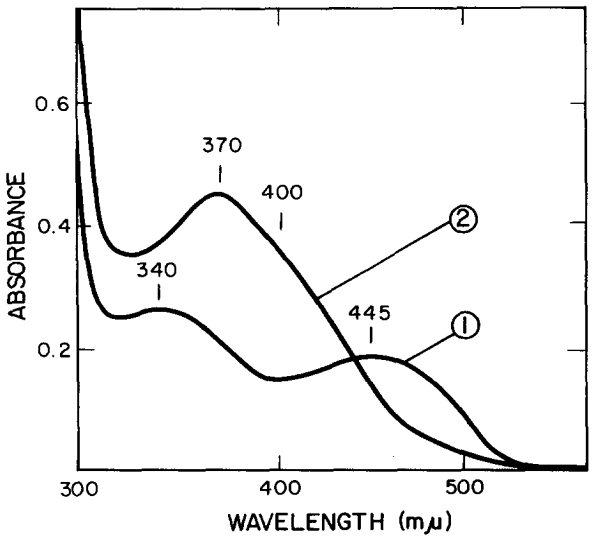

Fig. 1 Optical spectra of flavin peptides from monoamine oxidase. Light path, 1 cm; curve 1 in water, 2 in 6 N HCl.

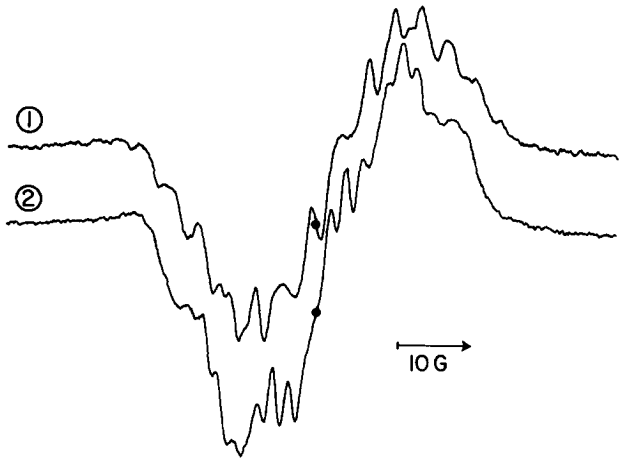

Fig. 2 ESR spectra of cation radicals of riboflavin (1) and of histidyl-8α-riboflavin (2) in 6 N HCl. The flavins (1 mM) were reduced with $TiCl_3$ and measured at room temperature with a Varian E-9 spectrometer at 9.47 GHz resonance frequency, 8 mW power, 0.25 G modulation amplitude, 100 KHz modulation frequency, 1 sec time constant, 8 min scanning time.

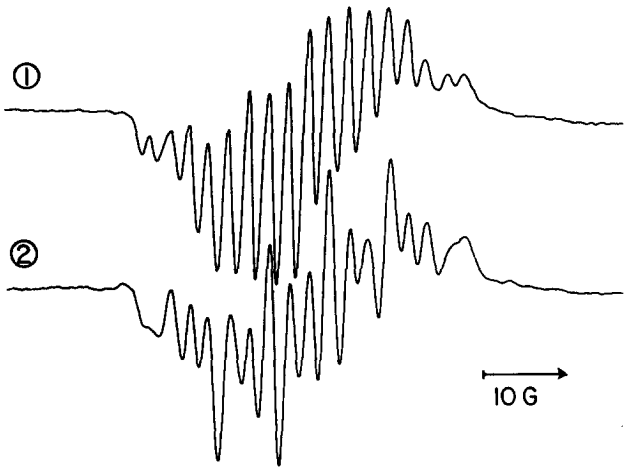

Fig. 3 ESR spectra of cation radicals of two flavin peptides derived from monoamine oxidase. The flavin concentration was 1 mM and conditions as in Fig. 2.

---

form by thermal or acid denaturation, and that a correspondence existed between the bound flavin content and monoamine oxidase activity on purification of the enzyme (16). This confirmed studies by Yasunobu (9,10) and Hellerman (11) and their coworkers that the flavin of mitochondrial monoamine oxidase is indeed covalently bound. The first question to be answered, therefore,

was the site of attachment of the peptide chain on the flavin ring system. Past studies in this laboratory, in collaboration with those of Ehrenberg and Hemmerich (1,2), have shown three parameters to be the most useful in assigning the site of substitution in this type of compound: optical spectra, ESR, and chemical stability. In each of these techniques reliance is made on the properties of model flavins substituted in known positions (for a review of the relevant studies on model flavins cf. ref. (1,2,17)).

Experience with the flavin of SD has shown that, as far as the optical spectrum is concerned, the most characteristic features are detected in the second (370 mμ) band of riboflavin in various states of protonation. In SD this is shifted to 345 to 355 mμ in the neutral and 370 mμ in the cation state of the oxidized flavin (1, 17). Literature reports on monoamine oxidase claim the presence of a 375 mμ band from difference spectra of the enzyme and absolute spectra of the flavin peptide in the neutral form (9-11) while a recent report shows a broad band at 350 mμ (12). The fluorescence excitation maximum, which should be relatively free from interfering impurities, has been variously reported to be at 360 to 375 mμ (10-12). The cation spectrum has not been examined.

We have found (Fig. 1) that partially purified preparations of the flavin peptide at the monophosphate level exhibit a clear maximum at 340 mμ, while the cation form shows a peak at 370 mμ, both as in SD. These facts suggest that the substitution is in the 8α-position.

This assignment is confirmed by the ESR hyperfine spectrum of monoamine oxidase radical cation. As reference patterns, Fig. 2 shows the hyperfine spectra of riboflavin and of histidyl-8α-riboflavin (SD-flavin) cation radicals. As previously reported (1,2,17), the total number of partly resolved hyperfine lines of riboflavin (14 lines, spacing = 3.8 G, and total spectral width = 49 G) changes dramatically upon substituting one main hyperfine coupling proton of the 8α-group with the bulky histidine to result in 20 lines, 2.3 G spacing, and 44 G total width. Fig. 3 shows the ESR hyperfine patterns of two flavin peptides isolated from monoamine oxidase in the cation radical

form. As may be seen, the spectra are well resolved, particularly near the center. As in the case of riboflavin and histidyl-8α-riboflavin, these spectra are asymmetric, with some resulting uncertainty in the determination of the exact number of hyperfine lines. The maximum number of lines possible is 20, as in histidyl-8α-riboflavin, the minimum 17, still considerably different from riboflavin itself.

The spacing between the hyperfine lines (2.3 G) is the same as in histidyl-8α-riboflavin, as is also the total spectral width (44 G), taking 20 lines as the basis of calculation. The differences in spectral intensities of the corresponding lines in the two samples of Fig. 3 might be the result of a small contamination of one of the two preparations with a radical arising from an impurity.

Taken together, the results in Figs. 1 and 3 strongly suggest that monoamine oxidase flavin, like SD flavin, is substituted in the 8α-position.

As regards chemical stability, the flavin of monoamine oxidase once again resembles SD-flavin: it is stable to heat and strong acids. Overnight hydrolysis of the peptide in 6 N HCl at 95° in vacuo did not shift the optical spectrum to that of normal flavins. Subsequent TLC chromatography in butanol-acetic acid-H_2O (4:2:2, v/v) and in NH_4OH-ethanol (7:3) revealed a ninhydrin-positive compound, migrating differently from riboflavin, FMN, and histidyl-riboflavin and well separated from free amino acids, showing that the 8α-position was still substituted with an amino acid. A secondary fluorescent spot was also detected. It is not clear whether the presence of two flavin spots indicates breakdown or incomplete hydrolysis.

Despite the many similarities with SD, the flavin of monoamine oxidase is different in one important respect: it shows the same fluorescence in the pH range of 3.5 to 8.0; hence substitution through an imidazole is ruled out. The fluorescence intensity is the same as that of riboflavin.

The normal pH-fluorescence behavior of monoamine oxidase flavin eliminates this enzyme as the one responsible for the low turnover number of SD in liver, which is based on the fluorometric determination of bound flavin

(8), since in such analyses the difference in fluorescence between pH 3.4 and 7.0 is used for calculations.

In other experiments flavin peptides obtained from purified preparations of the oxidase from beef and rat liver mitochondria were shown to contain the flavin in the dinucleotide form, since on acid hydrolysis to the mononucleotide level the fluorescence increased in the expected manner. This confirms the report (10) that the flavin is a dinucleotide.

ACKNOWLEDGMENTS

This research was supported by grants from the American Cancer Society (P 531), the National Science Foundation (GB 8248) and the U.S.Public Health Service (HE 10027). The authors are grateful to Prof. P. Hemmerich for the supply of synthetic histidyl-8α-riboflavin and to Dr. J.S. Hyde of Varian Associates for the ESR spectra.

REFERENCES

1. Hemmerich, P., Ehrenberg, A., Walker, W.H., Eriksson, L.E.G., Salach, J., Bader, P., and Singer, T.P., FEBS Letters, 3, 37 (1969).
2. Singer, T.P., Salach, J., Hemmerich, P., and Ehrenberg, A., in McCormick, D., and Wright, L., (Eds.) Methods in Enzymology, in press.
3. Walker, W.H., Salach, J., Gutman, M., Singer, T.P., Hyde, J.S., and Ehrenberg, A., FEBS Letters, 5, 237 (1969).
4. Walker, W.H., and Singer, T.P., J. Biol. Chem., 245, 4224 (1970).
5. Ghisla, S., Hartmann, U., and Hemmerich, P., Angew. Chem. Int. Ed., 9, 642 (1970).
6. Singer, T.P., Walker, W.H., Kenney, W., Kearney, E.B., Hemmerich, P., and Ghisla, S., in Ehrenberg, A. (Ed.), Structure and Function of Oxidation-Reduction Enzymes, in press.
7. Kenney, W.C., Walker, W.H., Kearney, E.B., Zeszotek, E., and Singer, T.P., Biochem. Biophys. Res. Communs., 41, 488 (1970).
8. Singer, T.P., in Florkin, M., and Stotz, E.H. (Eds.), Comprehensive Biochemistry, Vol. 14, Elsevier, Amsterdam, 1966, p. 127.
9. Nara, S., Igaue, I., Gomes, B., and Yasunobu, K.T., Biochem. Biophys. Res. Communs., 23, 324 (1966).
10. Igaue, I., Gomes, B., and Yasunobu, K.T., Biochem. Biophys. Res. Communs., 29, 562 (1967).
11. Erwin, V.G., and Hellerman, L., J. Biol. Chem., 242, 4230 (1967).
12. Gomes, B., Igaue, I., Kloepper, H.G., and Yasunobu, K.T., Arch. Biochem. Biophys., 132, 16 (1969).
13. Singer, T.P., Kearney, E.B., and Massey, V., Arch. Biochem. Biophys., 60, 255 (1956).
14. Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A., J. Cell. Biol., 32, 415 (1967).
15. Tabor, C.W., Tabor, H., and Rosenthal, S.M., J. Biol. Chem., 193, 265 (1951).
16. Gutman, M., Unpublished data.
17. Singer, T.P., Salach, J., Walker, W.H., Gutman, M., Hemmerich, P., and Ehrenberg, A., in Kamin, H. (Ed.), Flavins and Flavoproteins, in press.