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# THIOLS LIBERATE COVALENTLY BONDED FLAVIN FROM MONOAMINE OXIDASE

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SUMMARY Although it was recommended that 2-mercaptoethanol should be added to all buffers used in monoamine oxidase purification, purification of the enzyme in the absence of thiols has yielded double the amount of enzyme. In fact the presence of 0.1 M 2-mercaptoethanol or dithioerythritol-SDS[1] was found to liberate about 50% of the covalently bonded FAD. This observation is surprising since the covalently bonded flavin has been reported to be 8-α-cysteinyl-FAD. There is a thioether linkage between the flavin and the protein and mild reducing agents such as mercaptoethanol would not normally cleave the thioether linkage. The importance of the present finding is that the thiols may be used possibly to liberate suicide substrate-flavin adducts from the enzyme for structural studies without isolating pure flavin peptides. The flavin can be removed simply by treatment of the enzyme with 0.1 M thiol solution containing 1% sodium dodecylsulfate followed by chromatography on Sephadex G-25.

Bovine liver monoamine oxidase was first shown to contain covalently bound FAD by Igaue et al. (1). The elegant studies of Walker et al. (2) and Kearney et al. (3) concluded that the structure of this flavin in the enzyme was 8-α-cysteinyl-FAD. A number of suicide substrates of the enzyme which inactivate the enzyme by forming covalent adducts with the enzyme bound flavin include pargyline (4), 3-N,N-dimethylpropyne-1 (5) and phenylhydrazine (6). In order to determine the structure of the flavin adducts, it has been necessary to obtain and isolate pure peptides derived from the inactivated enzyme. Flavin release from the enzyme by chemical reagents would greatly simplify the task of determining the structure of the flavin adduct. Such reagents have been found and the procedure for liberating the flavin is discussed in the present report.

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[1] Abbreviations used is: SDS, sodium docecyl sulfate.

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## MATERIALS AND METHODS

Enzyme. Purified bovine liver monoamine oxidase was prepared by the procedure reported by Minamiura and Yasunobu (7) but 2-mercaptoethanol was omitted from all solutions used to purify the enzyme. 2-Mercaptoethanol, dithioerythritol and FAD were purchased from Sigma Chemical Co. Biogel A-0.5m was purchased from Bio Rad Labs. All common reagents used were of reagent grade quality.

Enzyme Determinations. The activity and specific activity of the enzyme were measured by the procedure of Tabor et al. (8) in which the oxidation of benzylamine to benzaldehyde is monitored at 250 nm. Protein concentrations were determined by the procedure of Lowry et al. (9) using bovine serum albumin as the standard.

Other Measurements. All spectral data reported were obtained in a Cary Model 14 Spectrophotometer. All pH measurements were made with the Corning Digital 112 pH meter.

## RESULTS AND DISCUSSIONS

Is Mercaptoethanol Needed to Improve the Yield of Purified Monoamine Oxidase. Minamiura and Yasunobu (7) recommended that 2-mercaptoethanol be added to all solutions used in the enzyme purification in order to increase the yield of enzyme. However, a recent purification of the enzyme in the absence of the thiol has resulted in a doubling of the yield of enzyme as shown in Table I. The increased yields of enzyme during purification are due to: (1) the initial washing of the mitochondria with Triton X-100 prior to disruption of the mitochondria plus the freezing of the initial homogenate which appears to liberate the enzyme from outer membrane fragments. However, it is still desirable to add $1 \times 10^{-5}$ M thiol to the purified enzyme since it not only maintains enzyme activity for about one month but also prevents the enzyme from precipitating. It should be noted that part of the enzyme has a very high specific activity (11,400) and was judged to be pure by the criterion of SDS-polyacrylamide gel electrophoresis, its molecular weight was determined to be 52,000. Although the remainder of the enzyme showed a lower specific activity it should nevertheless be suitable for certain types of enzyme experiments.

Spectral Properties of Purified Enzyme. The absorption spectrum of the purified enzyme (specific activity 11,400) showed a slight shoulder around 350 nm, a significant peak at 412 nm and a shoulder at about 450 nm

Table I. RESULTS OF ENZYME PURIFICATION (About 13 lbs Bovine Liver)[a]

| Step | | Volume | Activity (units/ml) | Protein (mg/ml) | Spec. Act. | Total units | Total Protein | Yield |
|---|---|---|---|---|---|---|---|---|
| 1. | C.5% Triton wash | 2,000 | 460 | 8.72 | 52.8 | $92 \times 10^4$ | 17,440 | |
| 2. | Homogenate | 2,000 | 2900 | 12.3 | 237 | $580 \times 10^4$ | 24,520 | 100 |
| 3. | 0.25-0.45 $AmSO_4$ ppt | 1,000 | 5250 | 5.95 | 882 | $525 \times 10^4$ | 5,950 | 91 |
| 4. | $CaPO_4$ Gel Eluate | 1,600 | 2250 | 0.90 | 2500 | $360 \times 10^4$ | 1,440 | 62 |
| 5. | DEAE Eluate | 377 | 9000 | 1.78 | 5056 | $339 \times 10^4$ | 671 | 59 |
| 6. | Ea Eluate | | | | | | | |
| | 0.2 M Buffer-0.2% cholate[b] | 138 | 8900 | 1.2 | 7295 | $123 \times 10^4$ | 168 | 21 |
| | 0.5 M Buffer-0.5% Cholate[c] | 78 | 16660 | 1.44 | 11528 | $130 \times 10^4$ | 112 | 22 |

[a] Prepared in 3 days starting from mitochondria to yield 43% of initial activity or 280 mg of enzyme from 5.8 kg of liver or 48.3 mg of enzyme per kg liver.

[b] At least 80% pure by SDS-disc electrophoretic test.

[c] Pure by SDS-disc electrophortic analysis.

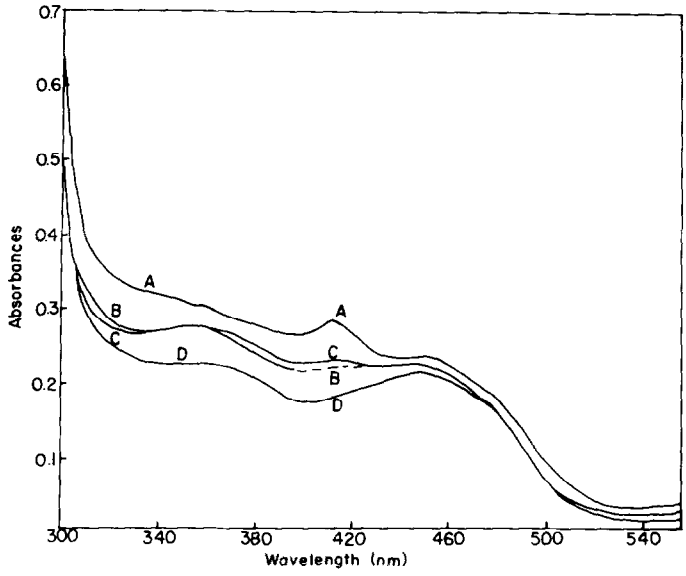
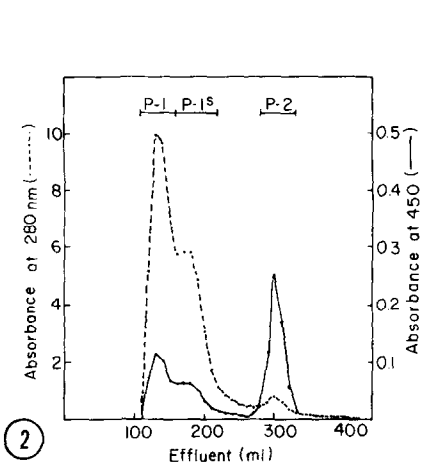



Figure 1. Visible absorption spectrum of monoamine oxidase, specific activity of 11,000. A, native enzyme at concentration of 1.78 mg per ml in 0.1 M potassium phosphate buffer, pH 7.4, which contained 0.2% potassium cholate B, identical to A except that the solution contained 1% SDS, enzyme: SDS ratio of 1:5.9. C, identical to B except that the solution was heated at 100° for 10 minutes. D, same as C except for the addition of mercaptoethanol to 0.1 M.

(Fig. 1). Although this 412 nm peak could be due to a heme containing contaminant this appears unlikely for the following reasons: 1) The enzyme has the highest specific activity of any preparation we have isolated thus far and shows a single band when examined by SDS-polyacrylamide gel electrophoresis. 2) The addition of dithionite does not bleach this peak as reported for heme-proteins. When the enzyme is heated in SDS, a slight shoulder is observed at 355 nm and the shoulder at 450 nm still persists but the peak at 412 nm is largely abolished. Therefore, the spectrum after this treatment resembles to a greater extent the spectrum of flavoproteins and the flavin content of the monoamine oxidase can be determined from the absorbance at 450 nm of the oxidized and reduced forms of the enzyme (10). The flavin content of the purified enzyme was found to be one mole of flavin per 110,000 g of enzyme.

Thiols Liberate Flavin from Monoamine Oxidase. Igaue et al. (1) showed that the FAD in monoamine oxidase was bonded to the enzyme covalently.



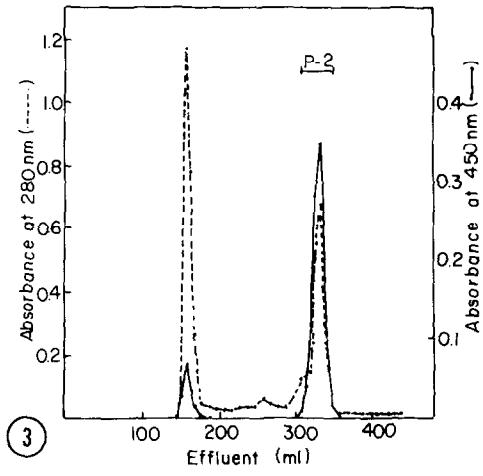




Figure 2. Separation of liberated flavin from monoamine oxidase by Biogel A-0.5m column chromatography. About 380 mg of enzyme in 0.02 M $NH_4HCO_3$ was first treated with 1.25% SDS-0.1 M mercaptoethanol (final concentrations) and then chromatographed on a 2.6 x 62 cm column of Biogel A-0.5m. The eluant was 0.02 M $NH_4HCO_3$.

Figure 3. Rechromatography of liberated flavin on Sephadex G-25. The concentration flavin obtained from the Biogel A-0.5m chromatography step was applied to a 2 x 113 cm column which was equilibrated and eluted with 0.02 M $NH_4HCO_3$.

Walker et al. have demonstrated that the flavin was bound as 8-α-cysteinyl-FAD (4). Much to our surprise, we have observed that the flavin in monoamine oxidase is liberated by thiols such as 2-mercaptoethanol and dithioerythritol. The enzyme was first dissolved in 1% SDS in 0.1 M potassium phosphate, pH 7.4. Sufficient 2-mercaptoethanol was then added to make the solution 0.1 M with respect to the thiol. The solution was then chromatographed first on a Biogel A-0.5m (Fig. 2) and then on Sephadex G-25 and the protein was separated from the flavin as shown in Fig. 3. From the absorbance, at 450 nm, it was determined that about 50% of the flavin had been liberated from the enzyme, while the remainder of the flavin was still attached to the enzyme although in a spectrally altered form. The liberated flavin was concentrated and the spectrum was recorded and compared with that of authentic FAD (Fig. 4), it showed maxima at 365 and 453 nm.

Concluding Remarks. The present investigation has shown that excess thiol liberated at least half of the flavin from monoamine oxidase. There-

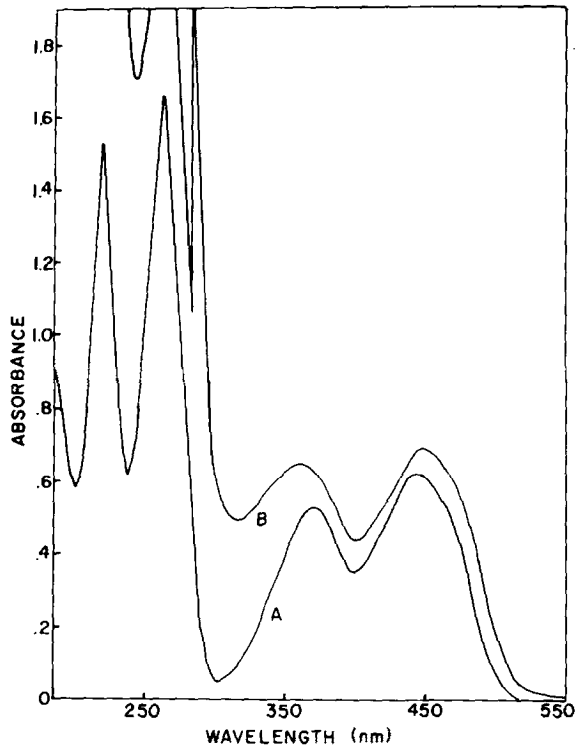



Figure 4. Ultraviolet-visible absorption spectrum of liberated flavin (P-2) from monoamine oxidase after Sephadex G-25 column chromatography. A, authentic FAD and B, flavin liberated from enzyme.

fore, caution must be excercized in its usage during enzyme purification. But the addition of thiol is still useful in storing the purified enzyme if the concentration is kept at $10^{-5}$ M. Thiol concentrations in the range of 0.1 M liberate the flavin in about 50% yield. Recent investigations have shown that a number of suicide substrates inactivate monoamine oxidase by reacting with the flavin (4-6). In order to characterize the flavin adducts, it has been necessary to use proteolytic enzymes to liberate flavin peptides which must then be purified. The present investigation has provided a simpler procedure for liberating the flavin, namely by the use of high concentrations of thiols such as 2-mercaptoethanol or dithioerythritol in denaturing solvents followed by Sephadex G-25 chromatography. Normally thioether linkages are not cleaved by mild reducing agents as thiols. There are several possible explanations for the cleavage of the flavin from the

enzyme. 1) The form in which the flavin is bound to the enzyme is not 8-α-cysteinyl-FAD but possibly a disulfide linkage between the flavin and protein or a thiohemiacetal linkage. 2) A more appealing explanation is that the structure of the bound flavin is instead 8-α-cysteinyl-FAD but that the S-C bond can be cleaved because of the activating effect of the benzenoid ring adjacent to the S-C bond.

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