Monoamine oxidase A from human placenta and monoamine oxidase B from bovine liver both have one FAD per subunit

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I present the first clear evidence that the protein:FAD ratio in human monoamine oxidase A and bovine monoamine oxidase B has an upper limit of 65 kDa and 57 kDa per FAD, respectively. To now it had been assumed that the protein:FAD ratio was 100–120 kDa to 1 FAD and that there was one FAD per two subunits which were assumed to be of the same size. For the present work the purity of monoamine oxidase A and monoamine oxidase B was improved over that previously achieved. Protein was determined by quantitative amino acid analysis and FAD content was measured by spectrophotometric titration of SDS-denatured enzyme with NaS_2O_4 standardized against riboflavin. The cause of the previous misassignment of the protein:FAD ratio was judged as having been due to the use of impure enzyme preparations. Knowledge of the correct protein:FAD ratio is important in devising cloning strategies for this enzyme, in understanding its structure, function, mechanism, and in the studies of its biosynthesis.

INTRODUCTION

Although monoamine oxidase [EC 1.4.3.4; amine:oxygen oxidoreductase (deaminating, flavin-containing)] has now been available in purified form from several sources for a number of years, there is a paucity of information on the primary and higher order structure of the two known isoenzymes. Based on colorimetric protein determinations, direct and indirect flavin determinations, amino acid analyses, and gel filtration experiments it has been suggested that monoamine oxidase B from bovine liver and other sources is composed of two 50–60 kDa subunits only one of which carries a covalently bound FAD [1–3]. More recently it was reported that monkey liver monoamine oxidase is composed of two subunits, although the FAD:subunit ratio was not addressed in this study [4]. White & Stine, however, recently observed monoamine oxidase activity in molecular sieving column eluates corresponding to the 50 kDa fractions [7,8] and preliminary data from this laboratory [5,6] suggested to us that the FAD:protein ratio was considerably higher than one FAD per 110 or 120 kDa of protein. Human monoamine oxidase A [9,10] and monoamine oxidase B [10] have recently been cloned and expression of cDNAs from a single subunit (with both human enzymes) gave catalytically active enzyme in a COS cell line [11]. These data suggest that monoamine oxidase is composed of a single type of subunit and it is likely that there is one FAD per subunit. To address this question I decided to investigate the question of FAD:protein ratio with monoamine oxidase A from human placenta and monoamine oxidase B from bovine liver by quantitative amino acid analysis and spectrophotometric titration of the covalent FAD cofactor. These analyses indicate that the upper limit of protein per FAD is 65 kDa and 57 kDa for monoamine oxidase A and monoamine oxidase B, respectively.

MATERIALS AND METHODS

General methods and materials

Standard amino acids, phenyl isothiocyanate, pyridine and triethylamine were from Pierce. Bovine serum albumin (BSA) was from Sigma (catalogue no. A-7638, lot no. 25F 9406). All other reagents were of the highest quality commercially available. Spectra were obtained with an HP diode array UV-vis spectrophotometer. H.p.l.c. was performed with a Beckman–Altex dual pump h.p.l.c. system having a Beckman 160 detector and a Shimadzu C-1RA integrating recorder. Spectrophotometric enzyme assays with benzylamine and kynuramine were done on a Perkin–Elmer Lambda3 scanning spectrophotometer as described previously [5,6] and anaerobic substrate/dithionite reductions to determine fraction of active enzyme were also as previously described [6]. Colorimetric protein determinations were done by the original method of Lowry *et al.* [12]; BSA concentration of stock solutions used to construct standard curves was determined by amino acid analysis as described below for monoamine oxidases. SDS/polyacrylamide-gel electrophoresis was by the method of Laemmli [13].

Enzyme preparation

Monoamine oxidase A and B were purified by previously published methods [5,6,14]. For amino acid analyses each enzyme preparation was further treated by DEAE-Sephacel CL-6B chromatography with all buffers containing 20% (w/v) glycerol and pH adjusted to 8. Monoamine oxidase A (13 mg) or monoamine oxidase B (11 mg; based on an assumed protein:FAD ratio of 60 kDa per FAD) were applied in wash buffer (see below) to columns (0.9 cm × 90 cm) packed with the above gel and previously equilibrated with 50 mM-potas-

sium phosphate or 20 mM-sodium phosphate, for monoamine oxidase A and B, respectively. The columns were then treated with 175 ml of wash buffer composed of 50 mM-potassium phosphate or 20 mM-sodium phosphate and 1 mM-D-amphetamine, 3 mM-2-mercaptoethanol and 0.2% Triton X-100 for monoamine oxidase A and B, respectively. Finally the columns were eluted with linear gradients of a total volume of 400 ml increasing from 50 mM- to 250 mM-potassium phosphate or 20 mM- to 250 mM-sodium phosphate, for monoamine oxidase A and monoamine oxidase B, respectively. Both of these gradients also contained a constant 0.8% octylglucoside, 1 mM-D-amphetamine and 3 mM-2-mercaptoethanol. The flow rates were about 20 ml/h, and 6 ml fractions were collected. In each case fractions containing enzyme activity were collected in three pools, leading edge, centre, and trailing edge. SDS gels were run on the pools to determine purity. The purest fraction for each enzyme was concentrated by ultrafiltration with an Amicon PM30 membrane. Aliquots of these pools were made 0.1% in SDS and dialysed against greater than 200 vol. of 20 mM-sodium phosphate, pH 7, containing 0.1% SDS and used for amino acid analyses. The optical spectra were measured to determine flavin concentration. A molar absorption coefficient of $11.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for FAD in the SDS-denatured enzyme was determined by anaerobic titration with $\text{Na}_2\text{S}_2\text{O}_4$ standardized against riboflavin, also by anaerobic titrations. Samples were made anaerobic in an anaerobic cuvette as previously described by cycling ten times between Ar and vacuum [6,15]. The published riboflavin absorption coefficient of $12500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used for calculations [16]. For one monoamine oxidase B sample the FAD content was also estimated by the method of Kearney [17] to assure that this method gives the same results as denaturation of the enzyme in SDS (see the Discussion).

Amino acid analyses

Protein samples (about 0.6 nmol or 35 μg) were hydrolysed for 24 or 48 h in 100 μl of 6 M-HCl at 110 °C in evacuated hydrolysis tubes, cycled ten times between Ar and vacuum prior to sealing. A mixture of 25 nmol each of standard amino acids (N, Q, S, G, H, T, A, R, P, Y, V, M, I, L, F and K) were hydrolysed in similarly evacuated tubes at 110 °C for 24 or 48 h and analysed in parallel to the monoamine oxidase samples. This method automatically corrects for time-dependent losses of hydrolysis-sensitive amino acids. Cysteine was determined separately as cysteic acid on lyophilized protein samples treated with 50 μl of performic acid reagent for 45 min or 1.5 h at 0 °C prior to acid hydrolysis (performic acid reagent was prepared by incubating 9 vol. of 88% formic acid with 1 vol. of 30% H_2O_2 at room temperature for 1 h; [18]). For internal standard 25 nmol each of α -amino-butyric acid, ϵ -aminohexanoic acid and norleucine was added to all samples prior to evacuation and hydrolysis. After hydrolysis, HCl was removed under vacuum for 30 min–1 h and amino acids were reacted with phenyl isothiocyanate (Pierce Chemical Corp.) as described by Henrikson & Meredith [19] except that the reaction was carried out for 15 min rather than 5 min. The derivatized amino acids were dissolved in 500 μl of buffer having the same composition as the starting buffer for the h.p.l.c. separation. Each analysis was usually done with 5 μl of these solutions (about 6 pmol of enzyme) on a reverse

phase Techsphere 3 μ , 4.6 mm $\times$ 150 mm column (Phenomenex) which was thermostated at 52 °C except for the cysteic acid analyses which were carried out at 40 °C. The elutions were accomplished with buffer systems and gradient adapted from Cohen *et al.* [20] and Henrikson & Meredith [19]. For cysteic acid determination, buffer A was 140 mM-sodium acetate titrated to pH 6.14 with H_3PO_4 ; for the other amino acids this buffer was back-titrated to pH 7.1 with NaOH. Solvent B was 60% acetonitrile and 40% water (v/v). The flow rate was 1 ml/min and effluent was monitored at 254 nm. The gradient used is expressed as %B and was as follows: $t = 0$, B = 8%, increasing to 18% in 0.4 min, then increasing to 25% in 0.6 min, then increasing to 31% in 1 min, and finally increasing to 51% in 6 min. Each segment was a linear ramp. All amino acids had eluted by 15 min and the column was recycled by increasing B to 100% in 0.5 min and holding for 9.5 min then returning to starting conditions by decreasing B to 8% in 2 min and holding for 13 min.

Tryptophan content of SDS-denatured (1% SDS) monoamine oxidase was estimated from the difference in protein absorption at 278 nm and the estimated absorption due to tyrosine, phenylalanine, and FAD at 278 nm. The molar absorption coefficients at 278 nm of each

Fig. 1. SDS/polyacrylamide-gel analysis of monoamine oxidase samples used for amino acid analysis

The gel is 7.5% acrylamide and 0.2% bisacrylamide, and was stained with Coomassie Brilliant Blue R-250. Protein concentration for loading was estimated for both enzymes from absorbance of samples at 456 nm and an estimated molecular mass of 60 kDa. (a) Monoamine oxidase A: lanes 1 and 2 are loaded with 1 and 5 μg of enzyme respectively, and lane 3 is Bio-Rad protein standards, in order of increasing molecular mass, ovalbumin, BSA, phosphorylase b, β -galactosidase and myosin, (b) monoamine oxidase B: lane 1, standards as in (a), lanes 2, 3, and 4 are loaded with 5, 2.5, and 1.25 μg of enzyme.

component were determined in the indicated solvents containing 1 % SDS and are: FAD, 23.0×10^3 (in water); Tyr, 1.26×10^3 (in 100 mM-HCl); Phe, 2.2 (in 100 mM-HCl); Trp, $5.35 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (in 100 mM-HCl). The assumption was made that there would be only small effects on the absorption coefficients of the amino acids in free solution as opposed to those bound in the protein [21]. This assumption seems also valid for FAD, as reduction with dithionite of the SDS-denatured enzyme has the appearance of a titration of free riboflavin while in the native enzyme the FAD clearly goes through the semiquinone [5,6].

RESULTS

Enzyme purification

After further chromatography on DEAE-Sepharose CL-6B the purity of both monoamine oxidase A and B improved, as was evident from amino acid analysis prior to and after this purification step. The SDS gel in Fig. 1 shows analytical samples after the last purification step. The purpose of this gel is to show integrity of the subunits. The human placental monoamine oxidase A which contained a 87 % fraction of substrate-reducible FAD prior to this chromatography retained a 70 % fraction of active protein after this treatment. In the case of monoamine oxidase B this chromatography lowered

the protein:FAD ratio by only 13 % as determined by quantitative amino acid analyses and spectral determination (see below) of FAD, but caused a 80 % loss of catalytic activity. The fraction of active enzyme was determined by anaerobic reduction of covalently bound FAD with substrate followed by its complete reduction with $\text{Na}_2\text{S}_2\text{O}_4$ [6]. An additional criterion of purity of these two enzymes is the ratio of the protein absorbance at 278 to the FAD absorption band at 452. This ratio was improved for monoamine oxidase A from 13.5 to 12, and for monoamine oxidase B from 13.4 to 12.4. These spectra were obtained on enzyme samples denatured with SDS for better spectral resolution.

Flavin quantification

The molar absorption coefficient for FAD of monoamine oxidase denatured with SDS was determined by anaerobic titration with $\text{Na}_2\text{S}_2\text{O}_4$ first standardized by anaerobic titration of riboflavin. A typical monoamine oxidase titration is shown in Fig. 2. A constant aliquot of dithionite was consumed without reduction of flavin at the beginning of all titrations, suggesting the presence of residual O_2 or some other reducible impurity. After this initial lag-phase in reduction of the flavin the system was well behaved. A molar absorption coefficient of $11.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was obtained (average of two

Fig. 2. Determination of absorption coefficient of denatured monoamine oxidase B

Glycerol was removed from 5 mg of monoamine oxidase B by centrifugation [5]. The enzyme pellet was taken up in 4 ml of 50 mM-sodium phosphate (pH 7.3)/1 % SDS. A 1 ml aliquot in an anaerobic cuvette was made anaerobic by 10 cycles of Ar and vacuum [15]. Aliquots of standardized dithionite (1.75 mM) were added from a Hamilton gas-tight syringe and spectra were recorded after each addition. Numbers on traces identify: 1, no addition; 2, 3 μl ; 3, 5 μl ; 4, 7 μl ; 5, 9 μl ; 6, 11 μl ; 7, 15 μl ; 13 μl addition is not shown for clarity, but is plotted in the inset. Inset: plot of absorbance change at 454 nm as a function of volume of dithionite added. Absorption coefficient was calculated from the usual relationship $A = \epsilon c l$. Calculations for this experiment: from the inset 11.2 μl of dithionite are used for the linear portion of the reduction. Therefore, 19.6×10^{-9} mol of FAD are reduced ($1.75 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} \times 11.2 \times 10^{-6} \text{ l}$). From trace 1, absorbance at 450 nm of sample is estimated at 0.22; therefore $\epsilon_{450} = 0.22 / 19.6 \times 10^{-9} \text{ M} \cdot \text{cm} = 11.2 \pm 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Table 1. Amino acid composition of human placental monoamine oxidase A and bovine liver monoamine oxidase B

Amino acids and FAD were determined as described in the Materials and methods section. For monoamine oxidase A average values were calculated from two sets of duplicate 24 and 48 h hydrolyses; no significant differences were observed between the 24 and 48 h hydrolyses. For monoamine oxidase B averages were calculated from triplicate 24 h hydrolysis; no significant differences were found for longer hydrolysis times. Cysteine was determined spectrophotometrically as described in [6]. Tryptophan was estimated spectrophotometrically as described in the Materials and methods section.

Residue	Monoamine oxidase A		Monoamine oxidase B	
	Residues/ molecule of FAD	Integer	Residues/ molecule of FAD	Integer
Asx	46.5 ± 0.4	47	37.2 ± 0.4	37
Glx	60.6 ± 1.0	61	49.5 ± 1.4	50
Ser	28.0 ± 1.8	28	29.4 ± 0.6	29
Gly	47.9 ± 1.4	48	42.7 ± 0.7	43
His	13.2 ± 0.3	13	13.6 ± 0.2	14
Thr	32.7 ± 1.0	33	26.9 ± 0.5	27
Ala	40.2 ± 1.4	40	32.1 ± 0.1	32
Arg	29.6 ± 0.4	30	27.2 ± 0.5	27
Pro	28.6 ± 0.3	29	28.5 ± 0.3	29
Tyr	27.9 ± 1.8	28	22.6 ± 0.6	23
Val	40.7 ± 1.3	41	33.4 ± 0.4	33
Met	15.8 ± 0.3	16	14.7 ± 0.7	15
Ile	34.6 ± 1.6	35	27.0 ± 0.1	27
Leu	47.0 ± 0.6	47	55.0 ± 0.4	55
Phe	20.1 ± 0.4	20	13.6 ± 0.1	14
Lys	37.7 ± 1.0	38	28.9 ± 0.7	29
Cys		8		8
Trp		14		16
Sum of residues		576		507
Calculated molecular mass (DA)		65 108		57 283

titrations). This absorption coefficient is in excellent agreement with that for free FAD, 11 300 M⁻¹·cm⁻¹ [13].

Amino acid analysis

Results of the amino acid analyses for monoamine oxidase A and B are shown in Table 1. The data clearly indicate that there is one flavin for 65 kDa or 57 kDa for monoamine oxidase A and monoamine oxidase B, respectively. Cysteic acid analysis gave 6–7 residues per molecule of flavin for monoamine oxidase B and A, respectively. These numbers are in general agreement with a value of 7 cysteine residues obtained by spectrophotometric cysteine titrations of reduced enzymes with dipyrindyl disulphide as previously reported [6]. Since FAD is attached through a non-titrable cysteine residue the estimated cysteine content is raised to 8 per molecule of FAD.

The spectrophotometric estimate of tryptophan gave values of 14.4 and 15.7 residues per molecule of FAD for SDS-denatured monoamine oxidase A and monoamine oxidase B, respectively. A similar method to estimate tryptophan has been reported for a large number of proteins by Spand & Witkop [21].

Both the values obtained for the number of cysteine

Table 2. Comparison of protein per molecule of FAD determined by amino acid analysis and by the method of Lowry with BSA as the standard

Quantitative amino acid analyses (AAA) and FAD determinations were carried out as described in the Materials and methods section; the first row is the results from Table 1. Absorption values for BSA are for 1% solutions at 280 nm and are from [23] (6.68) and [22] (6.17).

Method	Protein (DA)/molecule of FAD in:	
	Monoamine oxidase A	Monoamine oxidase B
AAA	65 108	57 283
Lowry, with BSA concentration for standard curves determined by:		
AAA	63 772	57 572
A _{1%} = 6.68	69 993	63 188
A _{1%} = 6.17	75 776	68 408

and tryptophan residues are also in substantial agreement with the number of these residues determined in a deduced amino acid sequence from a cDNA clone of human liver monoamine oxidase A [9,10].

Because in earlier work protein was determined by the method of Lowry *et al.* [12] it was important to compare results from this method with results from direct amino acid analysis of monoamine oxidase hydrolysates. BSA was used to construct standard curves and BSA concentration was determined by amino acid analysis. These analyses gave 15.8% or 10% lower protein content than would have been estimated from the published absorption coefficients of 6.17 [22] and 6.68 [23], respectively, at 280 nm for a 1% BSA solution. The molar ratios of amino acids were, however, in excellent agreement with the published data [22,24]. The average deviation of the molar ratios for each amino acid when compared with amino acid analysis by King & Spencer [22] was 3.7% (range -5% to +9%) and 3.4% (range -7% to +9%) when compared with the amino acid sequencing data by Brown [24]. The molecular masses for monoamine oxidase A and monoamine oxidase B determined by direct amino acid analysis were in best agreement with molecular masses determined by Lowry protein measurements when the BSA concentration used to construct the standard curves was determined by amino acid analysis or the high end of the published values for the absorption coefficients for BSA were utilized. These comparisons are summarized in Table 2, and show that the ratio of protein to FAD is 60–65 kDa/molecule of FAD.

DISCUSSION

I present here the first clear evidence that the protein:FAD ratio in human monoamine oxidase A and bovine monoamine oxidase B has an upper limit of 65 kDa or 57 kDa per FAD, respectively. SDS/polyacrylamide-gel electrophoresis indicates that this ratio corresponds to one FAD per subunit. FAD content was measured spectrophotometrically on enzyme samples denatured with SDS. Protein content was measured by quantitative amino acid analysis and by the method of Lowry *et al.* [12]. Both methods gave similar estimates of

protein. SDS gel experiments suggest that the difference in subunit size between human monoamine oxidase A and bovine monoamine oxidase B is about 3000 Da [6]. From the protein:FAD ratio determined here a subunit size difference between the two enzymes would be estimated as closer to 7000 Da. This discrepancy does not, however, alter the conclusion that there is one FAD per 60–65 kDa subunit. It does appear, though, that although the human enzyme looks pure by Coomassie Blue staining there are still up to 8% of protein impurities. This conclusion is also born out by the recent results from the cloning of human monoamine oxidase A which indicate that the exact subunit molecular mass of this enzyme is 59700 Da [9,10]. Further confirmation that the FAD:subunit ratio is 1:1 is due to the recent expression of monoamine oxidase catalytic activity from the cDNA of a single subunit of human monoamine oxidase (both monoamine oxidase A and monoamine oxidase B) in a COS cell line [11].

The protein:FAD ratio for fully active monoamine oxidase B, prior to the chromatography reported here, was 65 kDa per FAD (results not shown). After this chromatography the ratio fell to 57 kDa per FAD (13% decrease in protein) but the enzyme suffered a 80% loss in activity. The latter protein:FAD value corresponds closely to the observed subunit molecular mass determined by SDS gel electrophoresis with human monoamine oxidase A (59.7 kDa) as marker. The observed loss in enzymic activity during the final purification step does not recommend this method when active enzyme is required; however, it does not affect the conclusion that there is one FAD per approximately 60 kDa of protein which corresponds to the subunit molecular mass.

Previous reports in the literature suggested that the FAD content of monoamine oxidase is one FAD per 100–120 kDa. In particular for the bovine enzyme it was suggested in two reports that this enzyme is composed of two identical subunits of 55–60 kDa, only one of which contains covalent FAD [1,25]. These authors estimated FAD content by the spectrophotometric method of Kearney [17] in which trichloroacetic acid-precipitated protein was treated with trypsin and chymotrypsin to give an optically clear solution or by obtaining spectra after solubilization with 1% SDS. In our hands and in the previous reports both methods give comparable FAD quantification; the latter method was used in the present work. Two significant publications from another laboratory led to the notion that the protein:FAD ratio is 100 kDa of protein per FAD [2,3]. Curiously, although the specific activity of the enzyme had improved 2-fold in the later paper, the same protein:FAD ratio was concluded in both. In an attempt to obtain an understanding of the discrepancy between our protein:FAD ratio and the previously reported ratio, a preparation of bovine monoamine oxidase B with fully substrate-reducible FAD and a protein:FAD ratio of 65 kDa per FAD (this is the enzyme prior to the final chromatography reported here) was assayed and specific activity was determined under experimental conditions reported in the previous work [1–3]. The specific activity so obtained for this preparation was twice that reported for the enzyme used in the work of Minamiura & Yasunobu [1], and 1.41 times higher than that used in the work of

Chuang *et al.* [3]. These results are consistent with the idea that the lower protein:FAD ratio reported here is indeed the correct ratio.

The current results do not answer the question of whether there are one or more subunits per native monoamine oxidase or, if there are two subunits, are they identical or different in primary amino acid sequence. Knowledge that there is one FAD per subunit is important, however, in devising cloning strategies for this enzyme, in understanding its structure, function, and mechanism, and in the studies of its biosynthesis.

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