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COMPARATIVE STUDIES OF PURIFIED AND RECONSTITUTED MONOAMINE OXIDASE FROM BOVINE LIVER MITOCHONDRIA

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Monoamine oxidase, a strictly membrane-bound flavoenzyme, has been purified using a modified procedure recently developed. Probably similarly to other preparations known from the literature, the enzyme solubilizes to a clear suspension, which represents large clusters ranging in size from 5 to 50 nm containing appreciable amounts of residual lipids. The purified and reconstituted enzymes are inhibited differently by deoxycholate. In contrast to deoxycholate, Triton X-100 does not inhibit the purified enzyme, but rather disintegrates the lipid-enzyme clusters to the smallest active units. However, removal of the detergent leads to reconglomeration to larger lipid-enzyme aggregates. Using the irreversible destruction of the enzyme by deoxycholate as assay, reconstitution of the enzyme with exogeneous lipids has been studied. All basic enzyme properties, such as stability, maximal activity (V), Michaelis constant (K_m), pH- and temperature-dependence of the purified and reconstituted systems, are significantly different.

Introduction

Monoamine oxidase (amine : oxygen oxidoreductase (deaminating), EC 1.4.3.4) is assumed to exist in two forms, A and B. This is based primarily upon its substrate specificity and inhibitor sensitivity [1–4]. There is considerable experimental support for the existence of two different protein species [5,6]. However, the hypothesis that the enzyme exists in a single form which is modified by its specific membrane environment is also supported by many observations [3,7–9]. Previously, Houslay [10] obtained evidence that inhibitor sensitivity and substrate specificity are not strongly correlated but may be affected differently by the lipid environment.

Thus, studies on the enzyme in the membrane-bound form seem to reveal more valuable information on its physiological properties than do those on the solubilized enzyme. In addition, our present studies have shown that (a) the solubilized form is by no means a monomer, but a large aggregate with many residual lipids tightly bound, and (b) that solubilization procedures, such as sonication or treatment with various detergents, a prerequisite for purification, often cause irreversible damage to the enzyme by poorly understood mechanisms (cf. Ref. 11–14).

However, such studies have now become feasible with the development of procedures which yield highly purified and active monoamine oxidase [15], taking advantage of our experience with membrane-bound amphiphilic flavins [16,17].

In the present paper we describe the substitution of the residual lipids of the solubilized monoamine oxidase by two defined phospholipids and the effect on various basic enzyme parameters.

Materials and Methods

Materials. Benzylamine (801912) (recrystallized from ethanol as the hydrochloride to remove con-

taminating benzaldehyde), saccharose (7653) and sodium deoxycholate (6504) were obtained from Merck, Darmstadt, F.R.G. L-α-Phosphatidylcholine from egg yolk (P-5763), Ficoll type 400 (F-4375), poly(ethylene glycol) 6000 (P-2139), phospholipase C (P-7633) and octyl glucoside (O-8001) were purchased from Sigma, Dextran 2000 (18694) from Serva Heidelberg, phosphatidylserine (4-6004) from Supelco and Sephadex G-50 medium from Pharmacia. *Naja naja siamensis* venom was obtained from the Miami Serpentarium Laboratories (Haas Toxin); all other chemicals were of analytical reagent grade.

Purification of monoamine oxidase. Bovine liver mitochondria and other membrane fractions were prepared as described by Kearney et al. [18], and the isolation of monoamine oxidase was performed according to the procedure of Salach [15], except for the purification of phospholipase A from *Naja naja* venom, which was purified by chromatography on Sephadex G-50 [19]. Phospholipase activity was measured by an acidic assay [20], the pooled fractions were dialyzed against bidistilled H_2O for approximately 4 h to remove most of the $(NH_4)_2CO_3$, lyophilized, and dissolved again in 50 mM NaH_2PO_4, pH 7.2 (0.1 M NaOH).

Monoamine oxidase was stored at 4–10 mg protein/ml 50% (v/v) glycerol, containing 50 mM sodium phosphate, pH 7.2, at −10 to −20°C for several months; samples were taken without further purification.

Enzyme activity. Monoamine oxidase activity was measured spectrophotometrically by the method of Tabor et al. [21] as modified by Salach [15], except for the addition of Triton X-100. 1 unit of activity is defined as formation of 1.0 μmol of product per min. Approximately 7–10 μg protein were used per reaction mixture, the specific activity ranging between 1.4 and 3.0 U/mg protein.

Protein was determined by the biuret method [22] adding 50 μl of 10% (w/w) deoxycholate per ml to solubilize lipids. The purified enzyme was precipitated with 5% (w/w) trichloroacetic acid, the precipitate extracted with acetone and then redissolved in the biuret agent.

Kinetic studies. Kinetic constants, K_m and V, were determined by the standard assay as described above. Seven different concentrations were used for each determination, with the concentrations varied up to a 20-fold value. All experiments were performed with one preparation of monoamine oxidase. All data shown have been obtained within a 4 month time range. During this time the enzyme activity decreased significantly, which accounts for the different K_m and V values. However, each figure is consistant in itself.

Reconstitution with external lipids. $2.6 \cdot 10^{-5}$ mol of phospholipid (dissolved in 1 ml of $CHCl_3$/MeOH (9:1, v/v) for phosphatidylcholine and 0.4 ml of $CHCl_3$ for phosphatidylserine) were evaporated to dryness under vacuum at 0.67 Pa. The dry film was suspended in 5 ml of 50 mM sodium phosphate, pH 7.2, and sonicated for 120 min (phosphatidylcholine) or 90 min (phosphatidylserine) with a MSE-sonifier and microtip at a power setting of 70 W (20 kHz) using a waterbath at room temperature. Approximately $3 \cdot 10^{-9}$ mol of the purified enzyme (based on the molecular weight of 123000 [15]) were added and the mixture was sonicated for 4 min using a sonication bath [23] (Bandelin Sonorex RK 102/B) with an energy supply of 120 W at 35 kHz and thin-walled test tubes. All measurements were performed with freshly prepared lipid dispersions.

To perform the reconstitution by the addition of detergent, $2.6 \cdot 10^{-5}$ mol of phosphatidylcholine were dissolved in 1 ml of $CHCl_3$/MeOH (9:1, v/v) and evaporated to dryness under vacuum at 0.67 Pa. The dry film was suspended in 5 ml of 50 mM sodium phosphate, pH 7.2, and 1 ml of freshly prepared deoxycholate or octyl glucoside (final concentration, 0.82%) was added to give a clear solution within a half hour. After addition of approximately $3 \cdot 10^{-9}$ mol of the purified enzyme the solution was dialyzed at room temperature for 24 h against the buffer used.

Results

Reconstitution of monoamine oxidase with exogeneous lipid

There are essentially two major procedures available for the reconstitution of hydrophobic enzymes with external lipids: (A) Solubilization of the enzyme and the lipid(s) with detergents, such as Triton X-100, deoxycholate or octyl glucoside. Subsequent removal of the detergent by dialysis or

column chromatography results in the formation of intimate clusters of enzyme and lipid [24] or — in the ideal case — in the enzyme bound to single-shelled vesicles (unit membranes). (B) Sonication of homogeneous lipid-enzyme dispersions without detergent being present. Since monoamine oxidase turned out to be strongly inhibited by deoxycholate and, in contrast to the recent statement by Mimms et al. [25], by octyl glucoside also at the concentrations required, and since Triton X-100 is hardly removed from the enzyme-lipid suspension by dialysis (column chromatography would result in too high a dilution), method B remains the only choice.

Nevertheless, the distinct influence of both detergents used yielded valuable information on the reconstituted enzyme: our first attempt of an enzyme-lipid-reconstitution was based on method A. Within 48 h more than 99% of the deoxycholate originally inserted was removed by dialysis at room temperature (the experiments was performed with radioactively labelled deoxycholate, data not shown). However, about 50% of the monoamine oxidase activity was irreversibly lost.

This inhibition process was investigated in more detail. Fig. 1 shows the time course of monoamine oxidase inactivation in the presence of two concentrations of deoxycholate. After about 30 min incubation the activity assymptotically approaches certain distinct levels, indicating a 'static', stoichiometrically defined inhibition by deoxycholate. Fig. 2 depicts monoamine oxidase activity as a function of the deoxycholate concentration, again after a 30 min incubation. The data of Fig. 2 can be plotted in a Hill diagram, yielding a Hill coefficient not significantly different from 1.0. Measurements of activity of purified and detergent-treated monoamine oxidase (0.2%; 30 min; 30°C) yielded different K_m and V values in both cases (purified enzyme, K_m 0.19 mM, V 1.15 U; detergent-treated enzyme, K_m 0.36 mM, V 0.32 U).

Razin [26] proposed several criteria needed for a 'complete membrane solubilization', as required for a reconstitution experiment: one is that no sediment should be formed upon centrifugation of the solubilized membrane material at $100\,000 \times g$ for 1 h, which was met by our preparation of monoamine oxidase (appearing clear to the naked eye); another criterion given by Razin is only partially fulfilled: only 50% of the material is not excluded in the void volume of a Sepharose 4B column, indicative of 'clusters' ranging from 5 nm to more than 50 nm in size. This is consistent with the fact that addition of 0.002% Triton X-100 (molar ratio, 1 : 52) decreases the Rayleigh ratio by (maximal) 50%. However, the monoamine oxidase activity is unaffected by Triton X-100. On the other hand, deoxycholate shows just the opposite behavior: Rayleigh scattering is not affected at all by the detergent (up to a molar ratio of 1 : 12 000), but the enzyme activity is decreased (cf. Figs. 1 and 2).

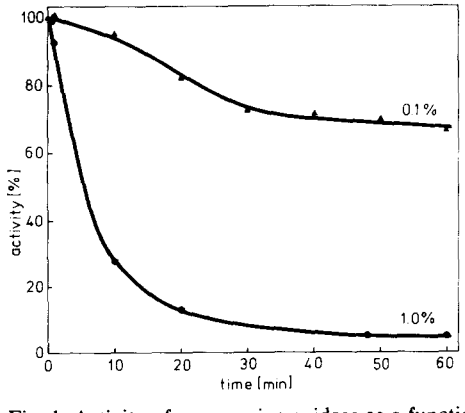

Fig. 1. Activity of monoamine oxidase as a function of incubation time at 30°C with deoxycholate at two concentrations, as indicated.

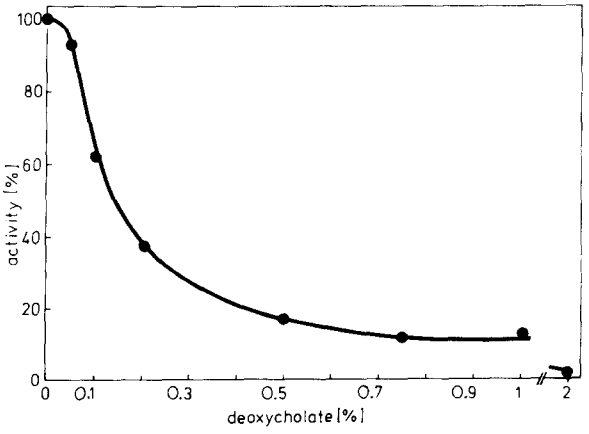

Fig. 2. Activity of monoamine oxidase as a function of deoxycholate concentration. Prior to measurement the enzyme was incubated for 30 min at 30°C with the various concentrations of deoxycholate given on the abscissa.

The capability of deoxycholate to inhibit monoamine oxidase activity can be adopted to monitor the process of reconstitution of the enzyme with lipids upon sonication. Fig. 3 shows the activity of monoamine oxidase added to phosphatidylcholine vesicles as a function of sonication time, in the absence and presence of (potentially inhibiting) deoxycholate. After 5 min of sonication the enzyme approaches a state of full protection from the inhibitor, which is taken to indicate completion of the reconstitution, i.e., equilibrium.

Fig. 4 shows our attempt (which failed initially) to perform the reconstitution of monoamine oxidase with exogeneous lipids using deoxycholate as dispersing agent and its subsequent removal by dialysis: 70% of the enzyme is irreversibly inactivated. This inactivation process is highly accelerated without external lipids present: about 5-fold for the initial rate. Both kinetics deviate significantly from a first-order time course.

The influence of two differently charged phospholipids (phosphatidylcholine and phosphatidylserine) on the activity of reconstituted monoamine oxidase is depicted in Table I.

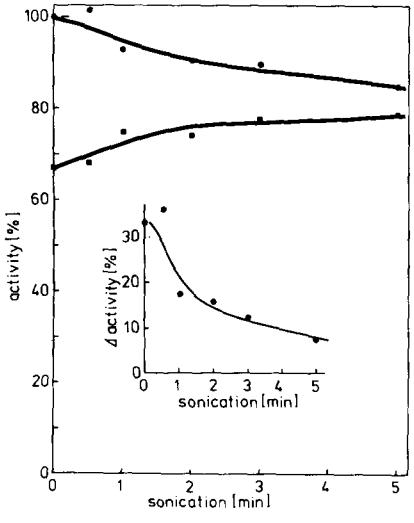

Fig. 3. Activity of monoamine oxidase, added to a pre-sonified phosphatidylcholine suspension, as a function of sonication time in the absence (●) and presence (■) of deoxycholate. Prior to measurement the sample was incubated with 0.8% deoxycholate for 10 min at room temperature; the inset depicts the averaged differences of the two curves.

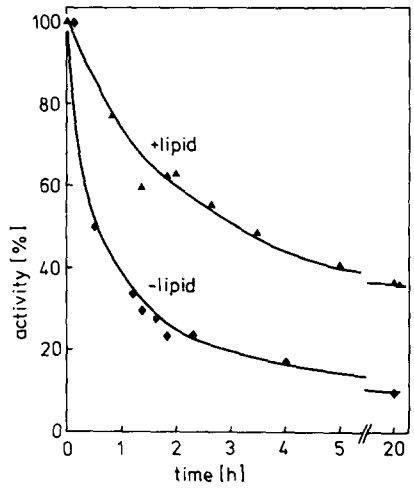

Fig. 4. Activity of purified (−lipid) and lipid-treated (phosphatidylcholine) monoamine oxidase as a function of dialysis time at 25°C.

Thermal stability of monoamine oxidase

Fig. 5 shows semilogarithmic plots of monoamine oxidase activity of purified and lipid-treated enzyme as a function of incubation time at 42°C. Under the latter conditions, the plot is essentially a superposition of two components. On this basis the analysis reveals first-order decay constants of approximately 0.01 min^{-1} (phosphatidylcholine and -serine, slow phase), 0.05 min^{-1} (phosphatidylcholine, fast phase) and 0.025 min^{-1} (phosphatidylserine, fast phase). At least 90% of the purified enzyme decays as a uniform species with a decay constant of 0.05 min^{-1}.

Strong differences are observed for the temperature-dependencies of enzyme activity of purified and lipid-treated enzyme (Fig. 6). Below 40°C the

TABLE I

KINETIC DATA OF PURIFIED MONOAMINE OXIDASE AND THE LIPID-TREATED ENZYME

Experiments were carried out under standard assay conditions.

	K_m (mM)	(V) (U)
Purified enzyme	0.28	1.5
Enzyme treated with phosphatidylcholine	0.28	1.1
Enzyme treated with phosphatidylserine	0.18	1.1

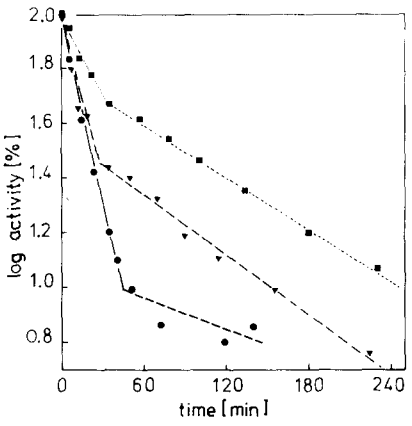

Fig. 5. Activity of purified (●) and lipid-treated monoamine oxidase as a function of incubation time at 42°C. ▼, Enzyme reconstituted with phosphatidylcholine; ■, enzyme reconstituted with phosphatidylserine.

same slope is observed in all cases, reflecting an activation energy of approximately 36 kJ/mol. However, for lipid-treated enzyme the temperature limit for thermal degradation is extended by about 10°C towards higher temperatures (note: the curves have been normalized at maximal activity. The absolute activities are 2.5, 2.3 and 1.8 U/mg protein for purified enzyme and the enzyme reconstituted with phosphatidylcholine and phosphatidylserine respectively.)

pH-dependencies of purified and lipid-treated monoamine oxidase

Fig. 7 shows the pH-dependence of monoamine

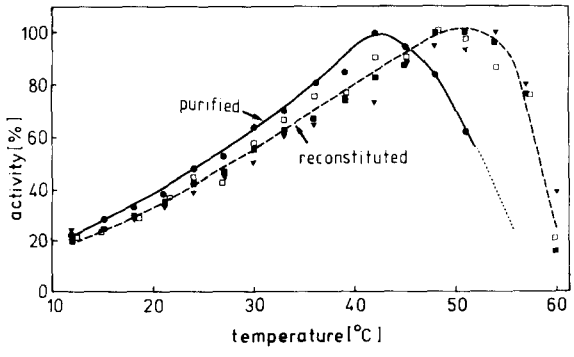

Fig. 6. Activity of purified (●) and lipid-treated monoamine oxidase as a function of temperature. ▼, Enzyme reconstituted with phosphatidylserine; ■, □, enzyme reconstituted with phosphatidylcholine.

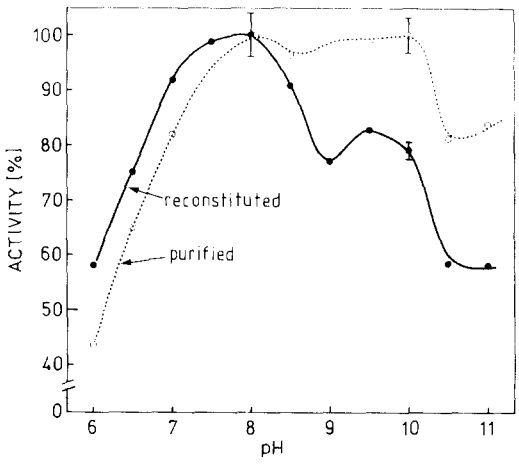

Fig. 7. Activity of purified (●) and phosphatidylcholine-treated (○) monoamine oxidase as a function of pH (reconstitution with phosphatidylserine reveals an identical pattern, curve not shown). Buffers used: pH 6.0–8.0, 50 mM sodium phosphate, pH 8.0–11.0, 50 mM borate.

oxidase activity of purified and lipid-treated enzyme. The curves have been normalized at maximal activity. Clearly, the pH-dependencies of monoamine oxidase activity are different for both conditions, almost independently of the specific lipid used.

Discussion

The purification of monoamine oxidase has been mostly performed by solubilization with detergents ([11,27–30]; method A, vide supra) rather than by sonication with or without detergent present ([8,31]; method B). Probably the most crucial point in all purification procedures of membrane-bound enzymes is their perfect solubilization, by damaging most of the membraneous structure. Small amounts of residual lipids or detergents generally cannot be removed from hydrophobic proteins in aqueous solvents, but can be minimized with respect to the enzyme activity. For example, for Salach's [15] best preparation Husain et al. [32] reported recently a molar lipid : enzyme ratio of approximately 5 : 1; however, our preparation, based on the same procedure, contains at least ten times more lipid.

The lipid environment of membrane-bound proteins appears to be an indispensible prere-

quisite for proper enzyme activity. Reports on reactivating and even enhanced activity of 'delipidated' monoamine oxidase by the addition of lipids are given by Huang [33] and Naoi and Yagi [34], but also the inhibiting influence of lipids [33,35] as well as the effect of detergent (assuming they are a specific type of lipid) [13,24] have been shown in numerous cases.

Information on the actual size of 'solubilized' monoamine oxidase can rarely be found in literature [12]. Probably due to particles badly defined in size and quality, the results obtained with monoamine oxidase reported in literature are nonhomogeneous [13,14,28,36]. In addition to our finding that Triton X-100 does not inhibit monoamine oxidase activity, strong variations are found in the literature [14,28,36]. This might be due to the fact that Triton X-100 is not a well-defined species [24] or that it cannot be efficiently removed by dialysis [24,26]. Removal by chromatography on Sepharose 4B yields particles excluded in the void volume (over 50 nm diameter).

Deoxycholate has a 2-fold impact on monoamine oxidase. The decrease in activity shown in Fig. 1 can be interpreted as a 'static', stoichiometrically defined inhibition of the enzyme (no reactivation possible); on this basis, Fig. 2 is taken as a 'binding curve'. The Hill coefficient of $n_H = 1.0$ indicates unspecific, non-cooperative binding; in contrast, SDS is known to bind cooperatively [24]. The initial 'lag phase' is significant and qualitatively comparable to data published by Achee and Gabay [14]. We assume that in the concentration range below 0.05% deoxycholate complexes are formed with residual lipids (for more detailed discussion and further literature cf. Ref. 24).

The opposite influences of Triton X-100 and deoxycholate could appear to indicate basically different mechanisms of solubilization of the enzyme, which we cannot understand on the basis of our present experiments.

The reduction of Rayleigh scattering by Triton X-100 in an asymptotic manner is taken to reflect degradation of larger monoamine oxidase particles (at least 50 nm as judged from the Sepharose 4B elution profile) into well-defined smaller units of activity.

There are several reports in the literature describing successful and fast reconstitution of various hydrophobic enzymes with exogeneous lipids simply upon incubation, without sonication or any detergent being present [33,35,37]. However, in the case of reconstitution of purified monoamine oxidase (method B) with exogeneous lipids, sonication turned out to be indispensible. On this basis, Figs. 3 and 4 are taken to reflect both exchange of intrinsic (residual) with exogeneous lipids and incorporation of the enzyme into larger membrane fragments.

Commonly, the role of phospholipids in monoamine oxidase activity is investigated by 'delipidating' the enzyme preparation and subsequent reconstitution with exogeneous lipids. This method requires either vigorous treatment with nonphysiological chemicals [34,35,38] or the use of lipases [29,33]. In both cases the resulting state of the enzyme appears to be poorly defined, which again is most likely the reason for the inhomogeneous results. The data shown in Table I give additional evidence that the catalysis of monoamine oxidase could be considered as a process depending on the microenvironment of the enzyme.

The thermostability of monoamine oxidase has been investigated in detail [8,29,39,40]. Enzyme stability and lipid content are strongly correlated, suggesting that the enzyme is protected from thermal denaturation by the membraneous environment. The course of enzyme activity as a function of incubation time (Fig. 5) can be interpreted by the assumption of a mixture of two distinct enzyme-lipid aggregates. Identical initial destruction rates of purified and phosphatidylcholine-reconstituted monoamine oxidase might be taken to indicate that in the latter system only part (approximately 40%) of the enzyme is reconstituted and the rest remains in the purified state.

The almost 10°C shift of the temperature optimum upon reconstitution (Fig. 5) can be explained if we assume that the reconstituted monoamine oxidase is better protected from temperature-degradation than is the purified enzyme [21,39,41].

The pH-optimum curve of the purified enzyme compared well with the data given by Williams [42]. Under the same experimental conditions the reconstituted enzyme exhibits a more pronounced dependency, with a sharp optimum at pH 8.0, rather than a constant activity of the purified

monoamine oxidase between pH 8.0 and 10.0.

McEwen et al. [31] and Williams [42] presented evidence that only the unprotonated amines will bind the protein. On this basis the K_m values of purified monoamine oxidase become pH-independent for pH values higher than the pK of the substrates. However, such constancy was not found for the enzyme when bound to mitochondrial membranes [40,43], in good agreement with our present results obtained from monoamine oxidase bound to artificial membranes (Fig. 7). This, in turn, is in good agreement with our previous observation that various flavin pK values may change by several units upon anchoring the chromophore within artificial membranes [17,44].

Summarizing, these data demonstrate that the reconstituted monoamine oxidase is a suitable model system, mimicking mechanisms in vivo and in natural membrane fragments under highly controlled conditions.

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