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Polystyrene microbridges used in sitting-drop crystallization release 1,4-diphenyl-2-butene, a novel inhibitor of human MAO B

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In the course of protein-structure determinations of the membrane-bound enzyme monoamine oxidase B (MAO B) by X-ray crystallography, a compound was found in the active site of the enzyme that consists of two phenyl rings separated by four C atoms. This compound was identified by chromatography and by mass spectrometry to be 1,4-diphenyl-2-butene and found to be a component of the polystyrene microbridges that are used in protein crystallization. This compound is present at a level of ~0.3 mg (~1.5 µmol) per microbridge and functions as a competitive inhibitor of MAO B with a K_i of 35 µ*M*. The presence of detergents in the crystallization solutions facilitates the extraction of this compound from the polymer medium.

1. Introduction

Crystallization of membrane-bound proteins is an area of protein structural biology that is plagued by many failures owing to difficulties in obtaining crystals suitable for diffraction studies. In those successful cases where suitable diffracting crystals are formed, the crystallization medium requires the presence of detergents at concentrations high enough to keep the protein in solution and to minimize the formation of amorphous aggregates. The sitting-drop vapor-diffusion technique is widely used for crystallization of biological macromolecules. The time required for vapor-diffusion crystal growth may require several weeks of contact between the crystallization microbridges, which are made of either polystyrene or polypropylene (the latter being compatible with organic solvents), and the detergent solutions. Therefore, apolar compounds from the plastic microbridges could, in principle, be extracted by these solutions during membrane-protein crystallization.

In our structural studies of human monoamine oxidase B (MAO B), an outer mitochondrial membrane-bound protein, we found an unexpected compound in the active site which interfered with the binding of weaker reversible inhibitors (K_i > 100 µ*M*). This communication documents its identification and the determination of its source, which was found to be the polystyrene crystallization bridges used in these experiments.

2. Experimental procedures

2.1. Materials

All chemical reagents used in analytical and synthetic procedures were purchased from Sigma–Aldrich. Human recombinant MAO B was expressed in *Pichia pastoris* and purified as described by Newton-Vinson *et al.* (2000). Materials used in protein crystallizations were purchased from Hampton Research Inc.

2.2. Crystallization conditions

MAO B crystals were grown by the sitting-drop vapor-diffusion method at 277 K. The precipitant solution consisted of 12%(*w*/*v*) PEG 4000, 70 m*M* lithium sulfate and 100 m*M* *N*-(2-acetamido)-2-iminodiacetic acid (ADA) pH 6.5. The protein solution contained 2 mg ml^{-1} MAO B, 8.5 m*M* Zwittergent 3-12 and 25 m*M* potassium phosphate buffer pH 7.5. Crystallization droplets were typically made by mixing 3.5 µl of protein solution with 3.5 µl of precipitant solution. Crystals belong to space group *C*222, with unit-cell parameters a = 131, b = 224, c = 87 Å and one MAO B dimer in the asymmetric unit. Data-collection, structure determination and refinement statistics for the X-ray analysis of MAO B in complex with 1,4-diphenyl-2-butene have been reported elsewhere (Binda *et al.*, 2003).

2.3. Analytical procedures and synthesis

MAO B oxidation of benzylamine was followed spectrophotometrically at 250 nm in 50 m*M* HEPES buffer pH 7.5 containing 0.5% reduced Triton X-100. Aliquots of a methanolic solution of the studied inhibitor (10 µl) were added to the assay cuvette containing buffer and substrate and incubated at 298 K before addition of MAO B (1 ml total volume). *N*,*N*-Dimethylformamide was used instead of methanol to dissolve 1,6-diphenyl-1,3,5-hexatriene. Electron-impact mass spectrometry (EI–MS) analysis was performed at the Emory University Mass Spectrometry facility in the

Table 1
Properties of fractions from silica-gel separation of the dichloromethane extract of a polystyrene microbridge.

Fraction	UV λ_{max} (nm)	EI–MS *m*/*z* (relative intensity)†	Identity
I	210, 254	208 (M^+, 28), 129 (11), 118 (11), 117 (100), 115 (33), 104 (49), 103 (10), 91 (57), 78 (17), 77 (19), 69 (23), 65 (23), 57 (40), 56 (19), 55 (12), 51 (20)	1,4-Diphenyl-2-butene ($C_{16}H_{16}$)‡
II	206, 240	279 (13), 211 (11), 208 (11), 207 (26), 206 (17), 167 (24), 149 (62), 137 (10), 129 (29), 128 (12), 123 (11), 121 (10), 113 (11), 112 (10), 111 (13), 109 (15), 105 (17), 104 (18), 97 (14), 95 (21), 93 (10), 91 (62), 85 (18), 83 (22), 81 (39), 79 (12), 77 (13), 76 (12), 71 (43), 70 (27), 69 (92), 57 (100), 56 (27), 55 (57)	Unknown
III	210, 262	208 (11), 207 (65), 206 (32), 178 (12), 130 (10), 129 (71), 128 (20), 115 (10), 105 (25), 91 (100), 79 (11), 77 (17)	Unknown
IV	206, 240	312 (M^+, 17), 208 (13), 207 (35), 194 (17), 129 (28), 128 (11), 117 (43), 115 (22), 105 (11), 104 (17), 103 (15), 92 (21), 91 (100), 78 (11), 77 (20), 65 (19), 57 (13), 51 (10)	1,4,6-Triphenyl-2-hexene ($C_{24}H_{24}$)‡

† Only peaks with relative intensity > 10 are listed. ‡ The number of C atoms was determined from the intensity ratio of M^+ and $(M + 1)^+$ and the number of H atoms was calculated from the molecular weight by subtracting the weight of the C atoms.

Department of Chemistry using a Jeol JMS-SX102/SX102A/E five-sector mass spectrometer.

2.4. Synthesis of *trans*-1,4-diphenyl-2-butene

1,4-Diphenyl-2-butene was synthesized from *trans*,*trans*-1,4-diphenyl-1,3-butadiene following a published protocol (Crotti *et al.*, 1984). The final product was recrystallized from ethanol (∼70% yield) and purified using a silica-gel column (1 × 30 cm, hexane as eluting solvent) to remove trace levels of 1,4-diphenyl-1,3-butadiene. The identity of the final product was determined by EI–MS [*m*/*z*, intensity; 208 (M^+), 27; 118, 10; 117, 100; 115, 24; 104, 19; 92, 10; 91, 44; 65, 13] and UV-absorption spectroscopy (λ_{max} = 210, 260 nm).

2.5. Extraction of polystyrene sitting-drop crystallization microbridges

Five microbridges (Hampton Research Inc., HR-312) were dissolved in 25 ml of dichloromethane. Polystyrene was precipitated by the addition of an equal volume of methanol. The precipitated polystyrene was twice re-extracted using the same procedure. The extracts were combined, dried using a rotary evaporator and the residue dissolved in 1 ml of hexane. This solution was analyzed by thin-layer chromatography (TLC, Merck 60 F_{254}), EI–MS and was also tested for MAO B inhibition. The extract was further fractionated using silica-gel chromatography as described above. Fractions absorbing at 260 nm were tested for MAO B inhibition and further analyzed by EI–MS.

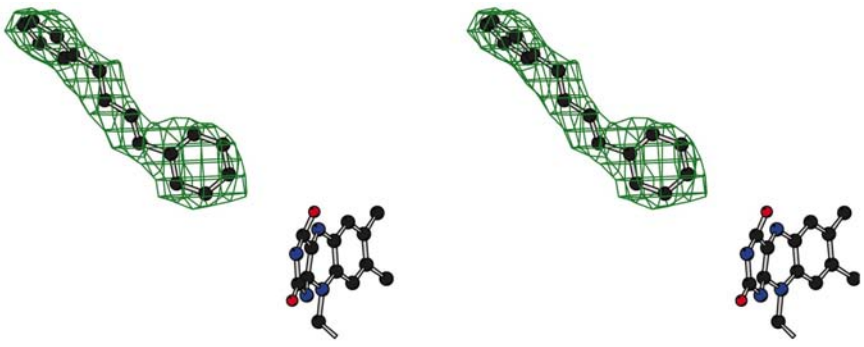

Figure 1
The unbiased $2F_o - F_c$ electron-density map at 2.3 Å resolution (shown in stereo at 1σ contour level) for the 1,4-diphenyl-2-butene inhibitor bound to MAO B (Binda *et al.*, 2003). The map was calculated before including the inhibitor atoms in the refinement calculation. The atoms of the flavin ring of FAD are shown to highlight the position of the inhibitor with respect to the prosthetic group. The flavin ring is in a highly distorted non-planar conformation as observed in all MAO B structures (Binda *et al.*, 2002, 2003). C atoms are in black, N atoms in blue and O atoms in red.

3. Results and discussion

3.1. Initial observations

On analysis of diffraction data from MAO B co-crystallized with the competitive inhibitor amphetamine ($K_i \simeq 250$ μ*M*), we found electron density in the active site of MAO B that was not consistent with amphetamine but resembled a 1,4-diphenylbutane analog (Binda *et al.*, 2003). As shown in Fig. 1, the electron density clearly shows two phenyl rings whose planes are orthogonal to one another and separated by four linking C atoms. Since this or similar compounds were not included in the crystallization medium, the origin of this material remained to be determined. Analysis of the Triton X-100 detergent used in the enzyme-purification procedure showed no compounds similar to a 1,4-diphenylbutane structure. Alternative sources such as the *P. pastoris* expression system also seemed unlikely. The polystyrene microbridges used in the vapor-diffusion crystallization experiments seemed a reasonable possibility since previous studies have shown the presence of diphenylbutanes in styrene and polystyrene (Midgley *et al.*, 1936; Zlatkis *et al.*, 1977). These bridges were the only labware made of polystyrene used in MAO B purification and crystallization.

3.2. Isolation and identification of 1,4-diphenyl-2-butene from polystyrene microbridges

The dichloromethane extract of the polystyrene microbridges contained several different low-molecular-weight compounds as shown by TLC analysis (Fig. 2) and competitively inhibited MAO B. The extract was preparatively fractionated on a silica-gel column in order to identify the low-molecular-weight compounds (Table 1). The fastest migrating band on the silica-gel column (I) was the only fraction that inhibited MAO B. I migrated with the same R_f value on TLC and exhibited the same UV-absorption spectral properties as synthetic 1,4-diphenyl-2-butene. The EI–MS of fraction I was determined to be identical to synthetic 1,4-diphenyl-2-butene (Table 1). The K_i value of I is estimated to be ∼50 μ*M* (concentration based on A_{260}), whereas the K_i value for synthetic 1,4-diphenyl-2-butene is 35 μ*M* (both exhibit competitive inhibition; Table 2). The presence of a small amount of II in I could explain both the observed difference in K_i values and the slight shift in the absorption maximum at 260 nm (Table 1). Comparison of K_i values for MAO B inhibition by a number of

Table 2
MAO B inhibition by analogs related to 1,4-diphenyl-2-butene.

Analog	MAO B K_i (μM)
1,4-Diphenylbutane	~100
1,4-Diphenyl-2-butene	35
1,4-Diphenyl-1,3-butadiene	7
1,4-Diphenyl-2,3-butandiol	>1000
1,6-Diphenyl-1,3,5-hexatriene	>1000

compounds related to 1,4-diphenyl-2-butene is shown in Table 2. Most are poorer inhibitors, with the exception of 1,4-diphenyl-1,3-butadiene, which exhibits an approximately fivefold higher K_i than 1,4-diphenyl-2-butene. None of the other analogs are detected in the polystyrene extract.

We estimate that approximately 0.5 mg of 1,4-diphenyl-2-butene can be isolated from five crystallization bridges. If ~30% recovery is assumed, each polystyrene crystallization bridge contains ~0.3 mg (~1500 nmol) of 1,4-diphenyl-2-butene. This level of 1,4-diphenyl-2-butene is sufficient to inhibit MAO B during crystallization and to interfere with the binding of reversible inhibitors that exhibit a lower affinity than 35 μM.

The presence of 1,4-diphenyl-2-butene in MAO B crystals is perhaps not surprising since it has been previously shown that 1,4-diphenylbutane analogs readily dissolve in detergent solutions (Ruiz & Aguiar, 1999). Material exhibiting absorbance at 260 nm is extracted when the microbridges are incubated in buffer containing lauryldimethylamine oxide and polyethylene glycol, whereas an extract with buffer alone yields no 260 nm absorbing material (data not shown). These data are in agreement with the results of Kolthoff & Graydon (1951), who showed that presence of alcohols increases the solubility of apolar compounds in detergent micelles. Since the enzymes' active site is found to be fully occupied by 1,4-diphenyl-2-butene in the crystals, we estimate that a lower limit of concentration of this compound in the crystallization medium to be at least twice the K_i value or 70 μM (or 490 pmol in 7 µl).

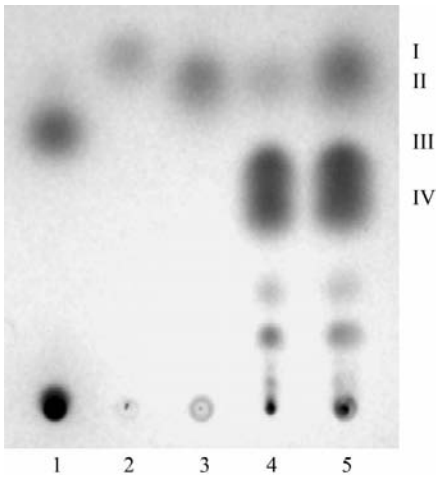

Figure 2
TLC analysis of the dichloromethane extract of polystyrene microbridges used in MAO B crystallization. Synthetic standards: 1,4-diphenyl-1,3-butadiene (lane 1, 120 nmol, R_f = 0.23), 1,4-diphenylbutane (lane 2, 200 nmol, R_f = 0.32), 1,4-diphenyl-2-butene (lane 3, 200 nmol, R_f = 0.26), polystyrene extract [lane 4, ~2% of extract from one bridge, R_f(I) = 0.26, R_f(II) = 0.25, R_f(III) = 0.20, R_f(IV) = 0.16] and polystyrene extract plus 1,4-diphenyl-2-butene (lane 5, same amounts as lanes 3 and 4) were separated on a silica-gel plate (Merck 60 F_{254}, 20 × 20 cm) using 15%(*v*/*v*) dichloromethane in hexane as a mobile phase. The bands corresponding to fractions from preparative silica-gel chromatography are marked with roman numerals.

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