Expression and processing of vertebrate acetylcholinesterase in the yeast *Pichia pastoris*

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In the methylotrophic yeast *Pichia pastoris*, we expressed the rat acetylcholinesterase H and T subunits (AChE_H and AChE_T, respectively), as well as truncated subunits from rat (W553stop or AChE_{TA}, from which most of the T-peptide was removed) and from *Bungarus* (V536stop, or AChE_{NAT}, or AChE_Δ, reduced to the catalytic domain). We show that AChE_H and AChE_T subunits are processed into the same molecular forms as *in vivo* or in transfected mammalian cells, but that lytic processes converting amphiphilic forms into non-amphiphilic derivatives appear to be more active in yeast. The production of glycosphosphatidylinositol (GPI)-anchored molecules (dimers, with a small proportion of monomers) demonstrates that *P. pastoris* can correctly process a mammalian C-terminal GPI-addition signal. Truncated rat and *Bungarus* AChE molecules, which exclusively generated non-amphiphilic monomers, were released more efficiently and thus produced more AChE activity. In the hope of increasing the

production of AChE, we replaced the endogenous signal peptide by yeast prepeptides, with or without a propeptide. We found that the presence of a propeptide, which does not exist in AChE, does not prevent the proper folding of the enzyme, and that it may either increase or decrease the yield of secreted AChE, depending on the signal peptide. Surprisingly, the highest yield was obtained with the endogenous signal peptide. For all combinations, the yield was 2–3 times higher for *Bungarus* than for rat AChE, probably reflecting differences in the folding efficiency or stability of the polypeptides. The Michaelis constant (K_m), the constant of inhibition by excess substrate (K_{ss}) and the catalytic constant (k_{cat}) values of the recombinant AChEs obtained both in *P. pastoris* and in COS cells, were essentially identical with those of the corresponding natural enzymes, and the K_i values of active-site and peripheral-site inhibitors (edrophonium, decamethonium, propidium) were similar.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is a remarkably efficient enzyme that hydrolyses acetylcholine into acetate and choline at a rate approaching the theoretical limit set by the diffusion of substrate [1]. The three-dimensional structure of *Torpedo* AChE has been solved by crystallography [2]. Unexpectedly, the active site is not directly accessible at the surface of the protein, but is localized at the bottom of a narrow catalytic gorge. This organization is paradoxical, since it appears to restrict the traffic of substrates and products in and out of the active site. The possible role of a local electric field, generated by charged residues of the enzyme, and of molecular movements that may open an alternative access to the active site ('back door' hypothesis), are being actively discussed [3–5].

The catalytic subunits of AChE are glycoproteins of 70–110 kDa, depending on the species. In vertebrates, AChE is encoded by a single gene, but alternative splicing generates subunits in which the same catalytic domain is associated with different C-terminal peptides that determine distinct post-translational modifications and quaternary associations [6]. Thus, the alternative H- and T-peptides define AChE_H and AChE_T subunits in *Torpedo* and in mammals. The H-peptide contains a cysteine, which may form an inter-chain disulphide bond, and a signal for cleavage and addition of a glycosphosphatidylinositol (GPI), so that AChE_H subunits generate GPI-anchored amphiphilic dimers. The T-peptide also possesses a free cysteine and forms an amphiphilic α -helix that allows a number of quaternary interactions: the AChE_T subunits exist as amphiphilic monomers (G_1^a), dimers (G_2^a) and tetramers (G_4^a), as well as non-amphiphilic tetramers (G_4^{na}); in addition, the tetramers may

form heteromeric associations with collagenic or hydrophobic proteins, which respectively anchor the enzyme in the basal lamina and in the cell membrane. The venom glands of *Bungarus fasciatus* contain a soluble monomeric form of enzyme [7,8], AChE_s, which possesses a distinct hydrophilic C-terminal peptide, without any cysteine [9].

In order to analyse the catalytic mechanism of AChE and the biosynthetic processes that lead to different AChE forms, AChE has been expressed in several systems. Active and correctly processed rat or other mammalian AChEs have been produced in COS cells [10–12], RBL cells [12], human HEK 293 cells [13] and baculovirus-infected insect cells [14]. *Bungarus* AChE was found to be expressed at a much higher level than rat AChE in COS cells [9]. Attempts to produce AChE in micro-organisms have not been very successful: in *Escherichia coli*, human AChE was accumulated as an inactive protein in inclusion bodies, and only a small proportion of active AChE could be recovered by re-folding and oxidation procedures [15].

Hoping to set up an efficient expression system to produce recombinant AChE, we used the methylotrophic yeast *P. pastoris*. This organism has several interesting characteristics: it can be grown conveniently to high cell density in a simple, inexpensive medium, and most importantly it can secrete proteins to very high levels, under the control of the efficient and tightly regulated promoter of the alcohol oxidase gene, AOX1. For example, invertase was secreted to 2.5 g/l in the medium [16], bovine lysozyme to 0.55 g/l [17] and recombinant tick anti-coagulant peptide to 1.7 g/l [18].

We presently report the results obtained by expressing rat and snake AChEs in *Pichia pastoris*. In the case of rat AChE, we analysed the molecular forms produced by post-translational

Abbreviations used: AChE, acetylcholinesterase; DS, detergent soluble; EU, Ellman unit; GPI, glycosphosphatidylinositol; LSS, low-salt soluble; MTP, O-ethyl-S-[(di-isopropylamino)ethyl]-methyl-phosphothionate; PHO1, acid phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C.

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processing of AChE_H and AChE_T subunits. We also expressed truncated subunits of rat AChE and of the snake *Bungarus*, which could only generate soluble monomers; in these constructions, we introduced stop codons after the ninth residue of the T-peptide of rat AChE_T (W553stop or AChE_{TA}), and immediately after the catalytic domain of *Bungarus* AChE (V536stop, according to *Torpedo* numbering), previously referred to as NAT [9] but called AChE_Δ here for the sake of uniformity. We also examined how the production of these monomers was modified by exchanging their own signal peptides for yeast signal peptides and by introducing propeptides which do not normally exist in AChE. We also compared the kinetic parameters of recombinant AChEs, obtained in *P. pastoris* and in the mammalian COS cells, with those of the natural enzymes.

MATERIALS AND METHODS

Materials

All reagents were purchased from Prolabo (Paris, France) or from Sigma (St. Louis, MO, U.S.A.). Restriction enzymes were from Biolabs (Ozyme, Montigny-le Bretonneux, France), and phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was from Immunotech (Marseille, France). *O*-Ethyl-*S*-[2-(di-isopropylamino)ethyl]-methyl-phosphonothioate (MTP) was kindly provided by the Centre d'Etudes du Bouchet (Vert-le-Petit, France). All plasmidic vectors were from Invitrogen (Leek, The Netherlands), except pYAM7SP8, which was a gift from Dr. Jan de Meutter (Corvas International, N.V., Belgium).

Construction of yeast expression vectors for AChE

Expression vectors for rat AChE

We constructed a series of *P. pastoris* expression vectors (Figure 1A), in which we inserted the coding sequences of rat AChE_H and AChE_T, as well as of a truncated subunit lacking most of the C-terminal T-peptide, AChE_{TA}, associated with various signal peptides and prosequences (Figure 1B). Site-directed mutagenesis was performed by using mutagenic oligonucleotide primers, as described previously [19].

The cDNAs encoding rat AChE_H and AChE_T were amplified by PCR from pEF-BOS vectors [20,21]. The amplification products were then subcloned in the pCRII vector (Invitrogen). After cutting by *EcoRI*, the AChE_H and AChE_T coding sequences were purified and inserted at the *EcoRI* site of the pHILD2 *P. pastoris* expression vector (Invitrogen), producing plasmids pHILD2/AChE_H and pHILD2/AChE_T respectively. In order to express a C-terminally truncated enzyme, we prepared the pHILD2/AChE_{TA} vector in which the W584 codon of the coding sequence of AChE_T was replaced by a TGA stop codon by site-directed mutagenesis.

Association of rat AChE_{TA} with various signal peptides and propeptides

We prepared different constructs in which the AChE_{TA} enzyme was associated with various combinations of signal peptides and propeptides, as illustrated in Figure 1(B).

To obtain pYAM7SP8/AChE_{TA}, the coding sequence of the catalytic domain with the truncated C-terminal T-peptide was amplified by PCR from pEF-BOS, using a forward primer located just after the signal peptide and a reverse primer which converted the W584 codon of AChE_T into a TGA stop codon and contained an *XbaI* site. The amplification product was then cut by *XbaI*, purified and ligated in phase with the sequence encoding the signal peptide of acid phosphatase (PHO1) and a synthetic propeptide into pYAM7SP8 [18], previously digested by *SstI* and *SpeI*.

For pPIC9/AChE_{TA}, an *EcoRI* site was introduced by site-directed mutagenesis just after the signal peptide of AChE_T in pHILD2/AChE_T. The mutated plasmid was then digested by *EcoRI* and *XbaI* and the insert AChE_{TA} was cloned into pPIC9, in phase with the pre-pro-sequence of the α -factor of *Saccharomyces cerevisiae*. In pPIC9 Δ pro/AChE_{TA}, the prosequence was removed by site-directed mutagenesis.

For pHILS1/AChE_{TA}, the *EcoRI*–*SalI* 3.6 kb fragment of pPIC9/AChE_{TA}, containing the coding sequence of AChE_{TA}, was ligated to the *SalI*–*EcoRI* 6.3 kb fragment of pHILS1.

For pHILD2/ α proAChE_{TA}, an *AscI* site was introduced by site-directed mutagenesis between the presequence and the prosequence of the α -factor in pPIC9/AChE_{TA} and an *MluI* site after the signal peptide of AChE in pHILD2/AChE_{TA}. The *AscI*–*SalI*

Figure 1 Schematic representation of the vectors used for expression of AChE in *P. pastoris*

(A) General structure of the plasmidic vectors. The circular map shows the promoter (5' AOX1) and the transcription terminator (TT, 3' AOX1) of the alcohol oxidase gene, the *HIS4* gene allowing selection by auxotrophic growth of *P. pastoris* without histidine, and a 3' AOX1 downstream region, allowing homologous recombination within the *AOX1* gene. The vectors used in this study encoded various pre- and pro-peptides (S), upstream of the insert encoding the catalytic domain of AChE. The restriction sites used for linearization of the vectors before transformation are also indicated. (B) Structure of the rat AChE constructs, indicating the structure of the C-terminal peptides, and the pre- and pro-sequences introduced upstream of the catalytic domain. The same combinations of pre- and pro-sequences were also introduced upstream of the *Bungarus* AChE_Δ sequence, except that we did not include a synthetic pro-sequence with the PHO1 signal peptide. The corresponding constructions are called by the same names, replacing AChE_{TA} by AChE_{NAT}.

3.8 kb fragment of pIC9/AChE_{TΔ} was then ligated to the *SalI*–*MluI* 6.4 kb fragment of pHILD2/AChE_{TΔ}.

Construction of expression vectors for *Bungarus* AChE

The pcDNA3/AChE_{NAT} vector encodes a truncated variant of *Bungarus* AChE, in which a stop codon was introduced at the end of the catalytic domain. It generates only a soluble, monomeric form of the enzyme. This form was previously called AChE_{NAT} after its C-terminal residues [9], but is now called AChE_Δ, since it is similar to the rat AChE_{TΔ} enzyme. The sequence encoding AChE_Δ was excised as a 1.7 kb *EcoRI*–*NotI* fragment and inserted between the *EcoRI* and *NotI* sites of pPIC9. The 3.6 kb *EcoRI*–*SalI* fragment was then ligated to the 6.3 kb *SalI*–*EcoRI* fragment of pHILD2 to generate pHILD2/AChE_{NAT}.

For pPIC9/AChE_{NAT}, an *EcoRI* site was introduced by site-directed mutagenesis just after the signal peptide of AChE in pcDNA3/AChE_{NAT}. The mutated plasmid was digested by *EcoRI* and *NotI* and the insert was ligated in pPIC9, after digestion by the same enzymes. In pPIC9Δ α pro/AChE_{NAT}, the α -factor prosequence was removed by site-directed mutagenesis.

The pHILS1/AChE_{NAT} vector was constructed by ligating the 3.5 kb *EcoRI*–*SalI* fragment of pPIC9/AChE_{NAT} into the 6.3 kb *EcoRI*–*SalI* fragment of pHILS1.

For pHILD2/ α proAChE_{NAT}, an *AscI* site was introduced by site-directed mutagenesis between the presequence and the prosequence of the α -factor in pPIC9/AChE_{NAT}, and an *MluI* site after the signal peptide of AChE in pHILD2/AChE_{NAT}. The *AscI*–*SalI* 3.7 kb fragment of pPIC9/AChE_{NAT} was then ligated to the *SalI*–*MluI* 6.4 kb fragment of pHILD2/AChE_{NAT}.

In all constructions, the engineered junctions were verified by sequencing.

Culture of *P. pastoris* and transformation, selection and induction of transformants

In this study, we used the GS115 *P. pastoris* strain (Invitrogen), which was grown in BMGY medium [1 % yeast extract, 2 % peptone, 0.1 M potassium phosphate (pH 7), 1.34 % yeast nitrogen base, 400 μ g biotin, 1 % glycerol] and induced in BMMY medium (like BMGY but glycerol was replaced by 0.5 % methanol), at 28 °C, in agitated 50 ml Falcon flasks. We also used a buffered minimal medium (BMM) which was identical with BMMY, except for the absence of yeast extract and peptone. Because AChE is irreversibly inactivated at low pH, expression of the recombinant enzyme was induced in a medium which was buffered at pH 7 with phosphate. Transformation of yeast cells was performed by electroporation in an Invitrogen Electroporator II, as described in the manufacturer's instruction manual (1500 V, 50 μ F, 100 Ω). All transformants were selected by the fact that they were His⁺, but integration of the plasmidic vector could occur either in the *HIS4* gene or in the *AOX1* gene. The plasmids used for transformation were digested by either *DraI* or *SalI*. Digestion with *DraI* yields fragments which can integrate by double or single crossing-over into the *AOX1* locus and can thus generate transformants in which the *AOX1* gene is disrupted (Mut^s) or transformants in which it remains active (Mut⁺). Linearization with *SalI* promotes integration into the *HIS4* locus, generating Mut⁺ transformants. Mut^s transformants were characterized by their slow growth or absence of growth on minimal medium plates [1.34 % (w/v) yeast nitrogen base, 400 μ g of biotin, 0.5 % (v/v) methanol]. Mut⁺/His⁺ transformants were selected by measuring the AChE activity secreted into the medium. Briefly, several His⁺ transformants were grown in 150 μ l of BMGY medium (in 96-well tissue-culture plates) and

then induced with methanol (0.5 %, v/v) for 24 h. After that time, secreted AChE was directly assayed in the supernatant (see below).

Selected Mut^s and Mut⁺ transformants were grown in 12 ml of BMGY medium. After 2 days at 28 °C, the cells were pelleted and resuspended in 7 ml of BMMY medium, buffered at pH 7. Every day, 0.5 % (v/v) of pure methanol was added to maintain induction, and an aliquot of the culture was removed to monitor the expression level of AChE in the cells and in the culture medium.

Preparation of yeast cell extracts

Cells were pelleted and washed once in ice-cold breaking buffer [50 mM sodium phosphate (pH 7.4), 1 mM PMSF, 1 mM EDTA and 5 % glycerol]. An equal volume of acid-washed glass beads (size 0.5 mm, Sigma) and ice-cold breaking buffer were added and the mixture was vortexed for eight periods of 30 s, separated by 30 s of incubation on ice. The sample was then centrifuged at 4 °C for 15 min at 10000 *g* and the supernatant, corresponding to the soluble fraction, was harvested. The pellet was resuspended in one volume of ice-cold breaking buffer supplemented with 1 % Triton X-100 and centrifuged in the same manner to obtain the detergent-soluble fraction.

Expression of rat and *Bungarus* AChE in COS cells

For comparison, rat AChE_{TΔ} and *Bungarus* AChE were expressed in COS cells, as previously described [9]. The secreted enzyme was recovered in the culture medium 4 days after transfection.

Extraction of AChE from rat brain

Rat brain was rapidly dissected and was first homogenized in a low-salt extraction buffer containing 50 mM Tris/HCl (pH 7), 10 mM MgCl₂ and 0.1 mg/ml bacitracin. The supernatant, defined as the low-salt soluble (LSS) fraction was recovered after centrifugation for 30 min, at 100000 *g* at 4 °C. The detergent-soluble (DS) fraction was obtained from the pellet by rehomogenizing in the same buffer supplemented with 1 % Triton X-100.

Analysis of AChE forms by sedimentation; treatment with PI-PLC

The molecular forms of AChE were analysed by sedimentation in sucrose gradients (5–20 %) without detergent, with 0.2 % Triton X-100 or with 1 % Brij-96. Centrifugation was performed at 36000 rev./min for 18 h at 7 °C in a Beckman SW41 rotor. The sedimentation coefficients were determined from the position of *E. coli* β -galactosidase (16 S) and *E. coli* alkaline phosphatase (6.1 S), which were included as internal standards in the gradients, by assuming a linear relationship with the fraction numbers. The AChE activity of each fraction was plotted as a function of the calculated sedimentation coefficients.

For PI-PLC treatment, AChE samples (50 μ l) were incubated at 30 °C for 1 h with 0.025 units of PI-PLC from *B. thuringiensis* (Immunotech), as described previously [10].

AChE activity assay and determination of kinetic parameters

AChE activity was measured by the colorimetric method of Ellman et al. [22], using acetylthiocholine as substrate. One Ellman unit (EU) is defined as the activity which induces an increase in absorbance of 1 per min in 1 ml of assay medium, with a pathlength of 1 cm. One microgram of rat AChE corresponds approximately to 30 EU and 1 μ g of *Bungarus* AChE to 60 EU [23].

The kinetic parameters of AChE were determined without purification using the culture medium or the cellular extract, in the case of recombinant AChE produced in *P. pastoris* and in COS cells, and an extract from rat brain in the case of natural AChE.

Catalytic constants (k_{cat}) were determined by titrating active sites with the irreversible inhibitor MTP [24]. Briefly, 20 μl of enzymic sample (containing about 5 EU/ml) were mixed with 20 μl of a dilution of MTP in phosphate buffer, using a range of MTP concentrations from 10^{-8} to 10^{-10} M. After 18 h at 4 °C, the residual AChE activity was assayed by the method of Ellman et al. [22] and plotted as a function of the dose of inhibitor; k_{cat} was deduced from the slope of the resulting line.

For determination of the Michaelis constant, K_m , and of the constant of inhibition by excess substrate, K_{ss} , assays were performed at 25 °C in 50 mM phosphate buffer, pH 7.4, containing 0.5 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.1 mg/ml BSA and a concentration of acetylthiocholine iodide ranging from 0.02 mM to 10 mM. K_m and K_{ss} were determined using the Haldane equation. Inhibition constants (K_i and αK_i) for edrophonium, decamethonium and propidium were determined in the same buffer, using three inhibitor concentrations and five substrate concentrations. The enzymic samples were incubated for 20 min with the inhibitor before addition of the substrate. The kinetic parameters were deduced from the linear representation of Lineweaver-Burk, and from secondary plots of the slope or ordinates at origin, using the Kaleidagraph software, as previously described [9].

RESULTS AND DISCUSSION

Expression of rat and *Bungarus* AChE in *P. pastoris*

Expression of rat AChE_H and AChE_T

We expressed rat AChE in *P. pastoris* using the pHILD2 vector (Figure 1A); pHILD2/AChE_H and pHILD2/AChE_T vectors were constructed by inserting the full coding sequences of rat AChE_H and AChE_T downstream of the *AOX1* promoter at the unique *EcoRI* restriction site (Figure 1B). In order to examine recombinant yeasts in which the plasmidic DNA was integrated in the *AOX1* and in the *HIS4* genes, the plasmids were linearized either by *DraI* or by *SalI* before transformation of the GS115 *P.*

Figure 2 Expression of rat AChE by *P. pastoris*

Release of AChE activity into the culture medium, upon induction by methanol, of *P. pastoris* expressing rat AChE_T (▲), AChE_H (■) and AChE_{TΔ} (●).

pastoris strain. Several transformants corresponding to both Mut^s and Mut⁺ phenotypes were isolated, as described in the Materials and methods section. They were grown in the BMGY medium and then induced at 28 °C for 8 days in the BMMY medium, buffered to pH 7 to maintain the enzymic activity.

Aliquots of culture were collected at different times after the beginning of induction, to examine the expression level of AChE in cell extracts and in the medium, as illustrated in Figure 2 in the case of Mut^s transformants. We found that similar amounts of AChE were produced by Mut^s and Mut⁺ transformants, indicating that the production of recombinant enzyme does not depend critically on the site of integration of the expression vector and on the phenotype of the clone. After 5 days, in 1 ml of culture containing about 5×10^9 cells, the cell extract contained about 6 EU of AChE_H and 4 EU of AChE_T, and only about 1 EU was recovered in the medium in both cases (Figure 2).

The fact that AChE_T was mainly associated with the cells contrasts with the results obtained previously with transfected COS cells, which released more than 70 % of the total AChE activity produced after 3 days (S. Bon and J. Massoulié, unpublished work). Thus secretion of AChE is less efficient in *P. pastoris* and the enzyme appears to be retained in the secretory pathway or in the periplasm. It is probably retained in the periplasm, since we obtained the same rate of acetylthiocholine hydrolysis with intact or broken cells, and this was not changed by addition of detergent (1 % Triton X-100).

Because a soluble monomeric form of AChE might be more easily released into the medium, we introduced a stop codon at position 584, removing the greater part of the T-peptide, and in particular the C-terminal motif that contains a free cysteine, CSDL. We analysed the expression of this truncated subunit, AChE_{TΔ}, in several Mut^s and Mut⁺ transformants, obtained with the vector pHILD2/AChE_{TΔ}. The total AChE activity produced was 5–6-fold higher than with AChE_T, and the secreted activity was 30-fold higher (Figure 2), although a significant fraction of the total activity remained associated with the cells. Since acetylthiocholine was hydrolysed at approximately the same rate by intact cells or after mechanical rupture by glass beads, the cell-associated activity appeared to be externalized, but trapped within the periplasmic space (results not shown). In this case, the level of AChE activity corresponded to about 1 mg of enzyme per litre of culture medium after 8 days of induction in BMMY at pH 7. The production of AChE was markedly affected by the pH and the composition of the culture medium: it was very much reduced below pH 6, and we could not detect any secreted activity when yeast extract and peptone were not included (minimal medium buffered to pH 6 or 7).

Expression of *Bungarus* AChE

We expressed a truncated form of *Bungarus* AChE (AChE_Δ) in the GS115 *P. pastoris* strain, using the natural signal peptide of the enzyme (pHILD2/AChE_{NAT}). Like rat AChE_{TΔ}, this form does not possess an H- or T-peptide, and generates only soluble AChE monomers [9]. We found that about 90 % of the AChE activity was secreted in the medium, corresponding to about 2 mg of AChE protein per litre.

Influence of the signal sequence on the production and secretion of rat and *Bungarus* soluble AChE

Although some heterologous proteins, such as α -amylase and lysozyme [17,25,26], have been secreted by yeast at a high efficiency with their own signal peptide, foreign secretion signals are often responsible for intracellular accumulation and degradation of the product. We therefore hoped to improve the

Table 1 Effect of signal peptides and propeptides on the production of secreted rat and *Bungarus* AChEs

Rat AChE_{rA} and *Bungarus* AChE_A enzymes were expressed with their own signal peptides, or with yeast signal peptides, with or without a prosequence, as shown in Figure 1(B). The values given (as EU/ml of medium; the cultures contained about 5×10^9 cells per ml) indicate the activity recovered in the culture medium, where it reached a plateau between 6 and 8 days after induction by methanol. The cells contained more activity in the case of *Bungarus* than in the case of rat, but this did not vary significantly with the signal peptide. ND, not done.

Signal sequence	AChE activity (EU/ml)	
	Rat AChE	<i>Bungarus</i> AChE
Native AChE signal peptide	20–30	100
PHO1	2	8
PHO1 + synthetic prosequence	1.5	ND
α -Factor preprosequence	15–20	40
α -Factor presequence	2	15–20
AChE signal peptide + α -factor prosequence	10	30–40

production and secretion of rat and *Bungarus* AChE in *P. pastoris* by using a yeast signal peptide. We exchanged the endogenous signal peptides of the rat AChE_{rA} and *Bungarus* AChE_A constructs with yeast pre- or prepro-sequences. We measured the AChE activity produced in the medium by Mut^s transformants, in which insertion occurred in the *AOX1* gene, so that all constructs could be compared in the same genomic context.

As shown in Figure 1(B), we used two secretion signals which have previously been found to be particularly efficient for secretion of heterologous proteins in *P. pastoris*: the preprosequence of the α mating pheromone of *S. cerevisiae*, and the signal peptide of *P. pastoris* PHO1. We also used the PHO1 signal peptide followed by a synthetic prosequence, which was found to increase 4–5-fold the expression and secretion of several proteins [18]. While classical signal peptides are usually cleaved by the enzyme signal peptidase during polypeptide synthesis, prosequences are cleaved in the Golgi apparatus by the KEX-2 protease at a Lys–Arg cleavage site, producing the mature protein. Natural AChE, however, does not possess such a prosequence, and its presence might affect the folding or the processing of the enzyme. We therefore studied several combinations of signal peptides, with or without a prosequence (Figure 1B).

We found that both rat and *Bungarus* AChE were expressed in an active form and secreted with all these constructs, but the yields were lower than those obtained with their native signal peptides (Table 1). We analysed the secreted protein by Western blotting with a specific monoclonal antibody (Bunga-46) [23] after concentration of the medium; as judged by the intensity of labelling, the amount of AChE protein corresponding to the same activity was similar for all constructs and for a purified venom preparation (results not shown).

This shows that the presence of a prosequence does not prevent the correct folding of AChE; it had a favourable effect on the yield of secreted activity in the case of the α -factor preprosequence, but not in the case of the synthetic prosequence associated with the PHO1 signal peptide. In the case of the α -factor preprosequence, as in the case of the endogenous signal peptide, more than 80 % of the activity was recovered in the culture medium, indicating that the enzyme was actively secreted. The results suggest that the rat and *Bungarus* signal peptides function quite efficiently in the translocation machinery of *P. pastoris*.

With a view to increasing the yield of secreted AChE, we introduced the α -factor prosequence after the endogenous signal peptides of rat and *Bungarus* AChEs. However, the presence of the prosequence reduced the production of both enzymes by a factor of 2–4. These results show that it is difficult to predict the effect of any combination of pre- and pro-peptides. It is likely that the immediate environment of the cleavage sites plays an important role in the processing.

As noted previously in the case of transient expression in COS cells [9], the production of *Bungarus* AChE was systematically higher than that of rat AChE, in spite of the fact that both enzymes were produced as soluble monomers. This suggests that the rate of biosynthesis and secretion largely depends on intrinsic properties of the protein itself, possibly related to its stability, or to the efficiency with which it folds into its active conformation. In effect, it has been shown that *Bungarus* AChE is able to renature into its active state after exposure to guanidinium hydrochloride, while AChE from *Torpedo* could not be reactivated under the same conditions (D. J. Kreimer, I. Silman, C. Bon and L. Weiner, personal communication).

Characterization of the molecular forms of AChE produced by *P. pastoris*

We analysed the molecular forms of active rat AChE in cellular extracts and medium from transformed yeast lines after 2 days of induction. The intracellular enzyme was first extracted without detergent (LSS extract) and subsequently in the presence of Triton X-100 (DS extract). The molecular forms were characterized by their migration in 5–20 % sucrose gradients, containing either Brij-96 or Triton X-100.

Molecular forms derived from rat AChE_H

In the case of AChE_H, the activity was mostly associated with the cells (90 %), while the culture medium contained only about 10 %. The secreted activity sedimented as two approximately equivalent peaks, at 4.5 S and 7 S, in the presence of either Triton X-100 or Brij-96, indicating that these two components correspond to non-amphiphilic monomers (G_1^{na}) and dimers (G_2^{na}) (Figure 3A).

The LSS extract, obtained by homogenizing the cells without detergent, contained about 40 % of the cellular activity. As shown in Figure 3(B), a major component of this fraction sedimented at 4.5 S in the presence of Brij-96 or Triton X-100 (results not shown), and was not affected by PI-PLC, indicating that it corresponds to a non-amphiphilic monomeric form (G_1^{na}). Similarly, a minor component sedimenting at 7 S could be interpreted as a non-amphiphilic dimeric form (G_2^{na}). In addition, the sensitivity to PI-PLC characterized GPI-anchored monomers (G_1^a), sedimenting at 3 S in Brij-96, and indicated the presence of GPI-anchored dimers (G_2^a), which co-sedimented with the G_1^{na} form in the presence of Brij-96.

In the DS fraction, containing about 60 % of the total activity, we observed a major component which sedimented at 4.5 S in the presence of Brij-96 and 6 S in the presence of Triton X-100 (results not shown), and was shifted to 6 S in both detergents after digestion by PI-PLC, indicating that it represents GPI-anchored dimers (G_2^a). It was accompanied by minor proportions of G_2^{na} and possibly of G_1^a (Figure 3C).

The molecular forms obtained in *P. pastoris* from AChE_H differ somewhat from those obtained in COS cells, because of the relatively high proportion of non-amphiphilic forms, G_1^{na} and G_2^{na} , which clearly reflect a lytic process, and the presence of GPI-anchored monomers, G_1^a . The major molecular form,

Figure 3 Sedimentation analysis of AChE forms obtained from *P. pastoris* expressing rat AChE_H

LSS and DS extracts from cells, as well as culture medium, were collected 2 days after induction by methanol. (A) Culture medium, (B) LSS extract, (C) DS extract. These fractions contained less than 10%, 40% and 60% of the total AChE activity respectively. They were analysed by centrifugation in sucrose gradients containing Brij-96 (○, △) or Triton X-100 (●), without treatment (circles, solid lines) or after digestion by PI-PLC (triangles, broken lines). The AChE activity of the gradient fractions is plotted on an arbitrary scale, as a function of the sedimentation coefficient, deduced by a linear relationship from the positions of the internal sedimentation standards. Broken arrows indicate the effect of PI-PLC, which converted GPI-anchored amphiphilic molecules into non-amphiphilic derivatives, presenting the same sedimentation coefficient in the presence of Brij-96 and Triton X-100. In the case of the medium, PI-PLC had no effect (results not shown). The nature of the molecular forms corresponding to each peak is indicated (arrows).

however, corresponds in both cases to GPI-anchored, cell-associated dimers (G_2^a), demonstrating that the C-terminal signal for cleavage and addition of a GPI anchor of rat AChE is efficiently processed by this yeast. The production of

Figure 4 Sedimentation analysis of AChE forms obtained from *P. pastoris* expressing rat AChE_T

The extracts were prepared as in Figure 3, and contained less than 10% (medium, A), 60% (LSS, B), and 40% (DS, C) of the total AChE activity 2 days after induction. The sedimentation profiles were obtained in the presence of Brij-96 (○) and Triton X-100 (●).

GPI-anchored monomers may reflect either reduction of the inter-subunit disulphide bonds, or proteolytic cleavage at an upstream site, removing the cysteine residues.

Although numerous examples of GPI-anchored proteins have been described in *S. cerevisiae*, this is, to our knowledge, the first case of a recombinant GPI-anchored protein produced in *P. pastoris* with a mammalian C-terminal GPI-addition signal.

Molecular forms derived from rat AChE_T

In the case of AChE_T, the culture medium contained less than 10% of the total activity, mostly non-amphiphilic monomers (G_1^{na}) sedimenting at 4.5 S, and a lower proportion of non-amphiphilic tetramers (G_4^{na}) sedimenting at 10.5 S (Figure 4A).

Figure 5 Sedimentation analysis of AChE forms obtained from *P. pastoris* expressing rat AChE_{TΔ}

The experiment was performed as in Figures 3 and 4. The sedimentation profiles obtained with a cell extract or with the culture medium were identical and were not affected by detergent in the gradients. We obtained similar profiles with *Bungarus* AChE_Δ (results not shown).

The LSS and DS extracts, representing respectively 60 % and 40 % of the cellular activity, contained very similar components (Figures 4B and 4C), mostly G_4^{na} , with small proportions of G_1^a and G_1^{na} . The only difference between these two extracts appeared to be the presence of a small G_2^a component in the DS fraction.

The molecular forms obtained in *P. pastoris* differ from those obtained in COS cells in the relative abundance of non-amphiphilic forms, i.e. the high proportion of G_4^{na} , in the cells. In addition, the quasi-absence of amphiphilic forms G_2^a and G_1^a , suggests that the amphiphilic α -helical part of the T-peptide may be removed by proteolysis, converting G_2^a and G_1^a into lytic derivatives G_2^{na} and G_1^{na} .

Molecular forms derived from rat AChE_{TΔ} and from *Bungarus* AChE_Δ

P. pastoris transformants expressing either rat AChE_{TΔ} or *Bungarus* AChE_Δ produced a single form of AChE, sedimenting at 4.5 S in the presence of Triton X-100 or Brij-96 (Figure 5), or without detergent (results not shown). We observed the same sedimentation pattern in the case of the secreted enzyme and cellular extracts, in which this enzyme could be entirely solubilized without detergent. These cells therefore produce exclusively non-amphiphilic monomers (G_1^{na}), in agreement with the absence of a C-terminal peptide which would allow the formation of oligomers.

Catalytic parameters of natural and recombinant rat AChE

AChE is a glycoprotein, and the nature of the glycans added by different cells may influence its catalytic properties. For example, AChEs extracted from bovine erythrocytes and lymphocytes, which appear identical in their immunological and hydrodynamic properties, differ in the binding of some lectins, and also present a significant difference in their K_m values [27]. Because the glycosylation performed by yeast cells differs from that of mammalian cells, we compared the catalytic parameters of recombinant rat AChE obtained in *P. pastoris* with a recombinant enzyme obtained in COS cells and with the natural enzyme extracted from rat brain. Both recombinant AChEs were secreted as monomers, produced by transfection with truncated rat AChE_{TΔ} or *Bungarus* AChE_Δ subunits, so that they were directly comparable. We analysed separately AChE from a cellular

Table 2 Kinetic parameters of native and recombinant AChE

K_m , K_{ss} and relative k_{cat} values were obtained with acetylthiocholine as substrate, as indicated in the Methods section, for native rat AChE (from brain) and *Bungarus* AChE (from venom), and for recombinant soluble monomers produced in COS cells and in *P. pastoris*. The k_{cat} values are given as a percentage of those obtained for the corresponding natural enzymes, determined in the same experiments.

Enzyme	K_m (μ M)	K_{ss} (mM)	k_{cat} (%)
Natural rat AChE	70 $\pm$ 4	25 $\pm$ 3	100
Recombinant rat AChE (COS cells)	63 $\pm$ 6	17 $\pm$ 3	120 $\pm$ 2
Recombinant rat AChE (<i>P. pastoris</i>)	74 $\pm$ 5	18 $\pm$ 2	115 $\pm$ 5
Natural <i>Bungarus</i> AChE	88 $\pm$ 5	17 $\pm$ 2	100
Recombinant <i>Bungarus</i> AChE (COS cells)	90 $\pm$ 5	18 $\pm$ 2	105 $\pm$ 5
Recombinant <i>Bungarus</i> AChE (<i>P. pastoris</i>)	86 $\pm$ 7	18 $\pm$ 3	100 $\pm$ 3

fraction and from the culture medium, which should not contain incompletely processed or incorrectly folded molecules [28,29]. We did not find any difference between the two extracts (results not shown).

In the case of rat brain, we analysed an LSS extract, which was enriched in the monomeric G_1^a form, and a detergent-soluble extract, containing mostly membrane-bound tetramers G_4^a . We did not observe any significant difference between monomers and tetramers, in agreement with previous studies which showed that the various oligomeric forms of AChE present the same turnover rate, k_{cat} [24].

The K_m , K_{ss} and k_{cat} values of the natural and recombinant enzymes appeared identical, within experimental error (Table 2). However, the recombinant enzymes were inhibited at somewhat higher concentrations than the natural enzyme by three distinct inhibitors: edrophonium, an active site competitive inhibitor, propidium, a non-competitive ligand of the peripheral site, and decamethonium, which interacts with both active and peripheral sites (Table 3). The difference between rat brain AChE and the corresponding recombinant AChE obtained in *P. pastoris*, with regard to the effect of decamethonium, is illustrated in Figure 6.

The *Bungarus* enzymes are markedly less sensitive to the peripheral-site inhibitor propidium than the rat enzymes, and approximately equally sensitive to the active-site inhibitor edrophonium and to the two-site inhibitor decamethonium. The kinetic parameters of the recombinant *Bungarus* AChE were similar to those of the AChE purified from *Bungarus* venom, which also consists of soluble monomers (Table 2). However, we found a similar difference in the inhibition parameters between the recombinant and natural enzymes as in the case of rat AChE_{TΔ} (Table 3). These differences are clearly unrelated to the quaternary structure of the enzyme, but might be related to the glycosylation introduced by the cells in which they are expressed.

Conclusion

We have shown that the yeast *P. pastoris* expresses vertebrate AChE in an active form, and that post-translational processing is very similar to that performed by vertebrate cells, in natural tissues or in transfected cell lines. In particular, the C-terminal GPI-addition signal of rat AChE_H subunits is correctly processed, so that *P. pastoris* produces GPI-anchored dimers, equivalent to those produced *in vivo*, in transiently transfected COS cells [20] or in permanently transfected RBL cells [12]. In the case of AChE_T subunits, *P. pastoris* produces monomers, dimers and tetramers, again like the mammalian cells, but monomers and

Table 3 Inhibition parameters of native and recombinant AChE

Values of K_i and αK_i were determined as shown in Figure 6. The values given are the means $\pm$ S.D. obtained from 3–5 experiments in the case of rat AChE, and a single experiment in the case of *Bungarus* AChE.

Enzyme	Propidium (μM)		Decamethonium (μM)		Edrophonium (μM)	
	K_i	αK_i	K_i	αK_i	K_i	αK_i
Natural rat AChE	2.5 ± 0.3	5.4 ± 1	5.5 ± 0.5	21 ± 2	0.39 ± 0.02	2.9 ± 0.3
Recombinant rat AChE (COS cells)	3.5 ± 0.7	6.1 ± 1	9.6 ± 0.8	26 ± 3	0.53 ± 0.02	5.5 ± 0.7
Recombinant rat AChE (<i>P. pastoris</i>)	4.2 ± 0.2	8.1 ± 0.7	10.8 ± 0.6	25 ± 2.5	0.64 ± 0.02	4.4 ± 0.3
Natural <i>Bungarus</i> AChE	29	355	9	26	0.62	4.4
Recombinant <i>Bungarus</i> (COS cells)	42	213	17	40	0.78	3.6
Recombinant <i>Bungarus</i> AChE (<i>P. pastoris</i>)	36	328	14	50	0.67	7.6

Figure 6 Inhibition of natural and recombinant rat AChE by decamethonium

One representative experiment for rat brain AChE (**A, B**) and recombinant rat AChE_T obtained in *P. pastoris* (**C, D**) is illustrated. (**A, C**) Lineweaver–Burk plots obtained in the presence of various concentrations of decamethonium ($\times$, 0; $\triangle$, 5×10^{-6} M; $\square$, 10^{-5} M; $\circ$, 2×10^{-5} M). (**B, D**) Secondary plots of the apparent K_m ($\square$) and slopes ($\blacktriangle$) as a function of the inhibitor concentration. The values of K_i and αK_i were deduced from the intersections of the lines obtained with the horizontal axis (Table 3).

dimers are mostly converted into non-amphiphilic forms, probably by cleavage of their C-terminal amphiphilic α -helical T-peptide, indicating an active lytic process. In spite of this conversion into lytic, non-amphiphilic molecules, much less AChE_T was released into the culture medium than in the case of COS cells. This appears to be largely due to retention within the periplasmic space. We obtained efficient secretion of rat AChE by removing most of the C-terminal T-peptide (AChE_{TD}). The *Bungarus* enzyme, which is naturally monomeric, was also efficiently secreted.

Since one of the aims of this work was to set up an efficient expression system for AChE, we compared various types of *P. pastoris* transformants. We found that the production of AChE was essentially the same in Mut^s recombinant yeasts, in which the

expression vector is integrated in the *AOX1* gene, and in Mut⁺ yeasts, in which it is integrated elsewhere, possibly in the *HIS4* gene.

We also analysed the effect of changing the secretion signal peptide of rat and *Bungarus* AChE, by introducing yeast secretion signals, either alone or combined with a propeptide, which is normally cleaved by the KEX2 peptidase in the secretory pathway. This showed that the presence of a prosequence does not prevent the proper folding of AChE and that it may increase or reduce the production of AChE, depending on the secretion signal. We observed considerable differences in the production of AChE with the various combinations of pre- and pro-sequences, resulting in the same order of efficiency for the rat and *Bungarus* enzymes, although rat AChE was expressed at a markedly lower

level than *Bungarus* AChE. It was surprising, and somewhat disappointing, to find that the highest AChE activity was obtained for both enzymes with their own secretion signal, without a prosequence. This shows that it is not possible to predict the efficiency of pre- and pro-sequences, independently of the nature of the secreted protein. In addition, the large and systematic difference observed between rat and *Bungarus* AChEs, in *P. pastoris* as in COS cells, suggests that the production of these proteins largely depends on their intrinsic properties, possibly on their stability or their capacity to fold into the correct active conformation. In any case, we did not find any evidence, by Western blotting, for the massive production of inactive rat or *Bungarus* AChE protein in the culture medium.

Our best production of rat AChE reached about 1 mg of secreted enzyme per litre of culture after 6–8 days of methanol induction. This yield is not high, compared with those obtained for other recombinant proteins in *P. pastoris*, but it might certainly be improved by increasing the number of integrated copies of the expression vector and the conditions of culture, notably the level of oxygenation. It might therefore be sufficient for a number of studies, if the enzyme could be easily concentrated and purified. For this reason, we attempted to induce secretion into a simple mineral culture medium, by omitting peptone and yeast extract. Unfortunately, we obtained no secretion of active AChE under these conditions. This study therefore demonstrates the capacity of *P. pastoris* to correctly synthesize and process vertebrate AChEs, but practical conditions allowing a high production of these enzymes remain to be defined.

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