Expression of active recombinant pallidipin, a novel platelet aggregation inhibitor, in the periplasm of *Escherichia coli*

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The platelet aggregation inhibitor pallidipin is a protein present in the saliva of the blood-sucking triatomine bug *Triatoma pallidipennis*. Expression of recombinant pallidipin in the periplasm of *Escherichia coli* was achieved by placing its coding sequence downstream of the alkaline phosphatase (APase) or *trc* promoter in frame with bacterial leader peptide DNA sequences derived from APase or from the periplasmic form of cyclophilin (Cph). In each case the DNA sequence of mature pallidipin was merged to the leader peptide coding part, either directly, or while introducing additional amino acids, in order to assess their influence on the activity of the leader peptidase and on the biological activity of the recombinant protein. All tested constructs gave rise to abundant periplasmic expression of pallidipin, which was then purified by a combination of cation- and anion-exchange chromatography followed by size-exclusion gel chromatography. Recombinant pallidipin had the expected molecular mass (~ 19 kDa) and was correctly processed, as demonstrated by SDS/PAGE and N-terminal amino acid sequencing. The highest expression levels were obtained with the three APase-derived expression plasmids. Platelet aggregation tests revealed that *E. coli*-derived pallidipin was fully active, with an IC_{50} of 33–89 nM, comparable with that of the native protein, except when an additional N-terminal lysyl-isoleucyl dipeptide was present, which resulted in an IC_{50} more than ten times higher.

INTRODUCTION

Pallidipin, a 19 kDa protein from the saliva of *Triatoma pallidipennis*, specifically inhibits collagen-induced platelet aggregation by an as yet unidentified mechanism [1]. Cloning of pallidipin cDNA and expression in baby hamster kidney cells helped to define some of the unique biological specificities of this polypeptide, which could be of interest as an anti-thrombotic agent [1]. Detailed studies on the mode of action of pallidipin and *in vivo* experiments have, however, been restricted so far by the scarcity of the material.

In order to provide the means for a rapid and economical production of pallidipin, we set out to devise and test a variety of expression constructs based on inducible promoters and leader peptides derived from alkaline phosphatase (APase) and periplasmic cyclophilin (Cph), two proteins located in the *Escherichia coli* periplasm.

APase is synthesized in *E. coli* as a precursor protein, of which the 21-amino-acid-long leader sequence is clipped off by the leader peptidase in the course of translocation across the bacterial inner membrane into the periplasmic space [2]. Its biosynthesis is regulated by the phosphate concentration of the culture medium [3], and export of heterologous gene products placed downstream of the APase promoter is achieved by growth in low phosphate concentrations [4–6].

Cph was originally described in eukaryotes as an abundant cytoplasmic protein [7] but later work revealed the existence of a whole family of Cph-related sequences [8,9], including a secreted form [10–12]. More recently, a periplasmic form of Cph was discovered in *E. coli* [13–15]. It is preceded by a characteristic 24-amino-acid-long leader sequence which triggers translocation of the protein into the periplasmic space [15].

Since severance of the leader peptide might improve expression levels and is also desirable to obtain a fully active mature form, the amino acid residues surrounding the desired cleavage site were carefully selected. Even though extensive site-directed mutagenesis studies have permitted the identification of recurring patterns, there would seem to be no absolute sequence requirement [16–20]. What is more, the sequence of the coupled mature region might well affect the processing and final destination, implying that leader peptides are not necessarily interchangeable [5,21–24]. Different expression constructs were therefore engineered, where the APase or Cph leader peptides were fused either directly to mature pallidipin or with intercalating additional amino acids likely to favour efficient processing.

In this report we compare the influence of two different promoter/leader peptide combinations and of the leader peptidase recognition sequence on the expression of recombinant pallidipin in *E. coli* by analysing a variety of parameters including overall yield, cleavage site, compartmentalization and biological activity.

EXPERIMENTAL

Materials

Cloning of pallidipin cDNA has been described [1]. The expression plasmid pSB/pho is derived from pSB94 [25,26] with additional *Sma*I and *Xma*I sites downstream of the leader peptide coding part, and was a kind gift from Dr. W. Boidol (Schering

Abbreviations used: APase, alkaline phosphatase; CF, cytoplasmic fraction; Cph, cyclophilin; IPTG, isopropyl β-D-thiogalactopyranoside; SN, supernatant.

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AG, Berlin). The pKK233-2 plasmid was obtained from Pharmacia LKB. *E. coli* strains E15 [27] and BL20 [28,29] were donated by Drs. W. Boidol and I. Kuhn (Berlex Biosciences, Richmond, CA, U.S.A.) respectively. Restriction endonucleases were purchased from Boehringer Mannheim, T_4 DNA ligase and mung bean nuclease from Stratagene. *Taq* polymerase and the PCR kit were from Perkin–Elmer Cetus. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. The sequencing kit (Sequenase) was from USB and [^{35}S]dATP[αS] (specific radioactivity > 1000 Ci/mmol) from Amersham. SeaPlaque low-melting-point agarose was from FMC, isopropyl β-D-thiogalactopyranoside (IPTG) from Gibco BRL. PMSF, Nitro Blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and the anti-(rabbit IgG)-conjugated APase were from Sigma. Collagen was purchased from Hormon Chemie. The polyvinylidene difluoride sheets (Immobilon) were from Millipore and the nitrocellulose BA 85 sheets from Schleicher und Schüll.

Construction of the expression vectors

Cytoplasmic expression was tried first. New convenient restriction sites (underlined in the sequence; the specificity is indicated in parentheses) were introduced, flanking the region coding for mature pallidipin, using PCR. The following sense and antisense primers were employed: cyt1, 5′-GCGATACCATGGAGGAG-GTGGCAACATGGAAGAATGCGAACTCATGCCACC-3′ (*Nco*I) and cyt1′, 5′-GCGATAAAGCTTATTACTTCATG-TTATCAC-3′ (*Hin*dIII). The template DNA (1 μg of pallidipin-1 cDNA plasmid) [1] was amplified by PCR in 15 cycles of 1 min at 94 °C, 1 min 30 s at 49 °C and 2 min 30 s at 72 °C. The amplification product was purified on a 1% low-melting-point agarose gel, digested with *Nco*I and *Hin*dIII before subcloning into pKK233-2, using T_4 DNA ligase.

For the periplasmic expression constructs, two series of vectors were prepared. The first was based on the pSB/pho plasmid. The following sense primers, pho1, 5′-GCGATATCGCGAAGAAT-GCGAACTCATG-3′ (*Nru*I), pho2, 5′-GCGATACCCGGGA-AGAATGCGAACTCATG-3′ (*Sma*I), and pho3, 5′-GCGA-TATCGCGACGAAGAATGCGAACTCATG-3′ (*Nru*I), and antisense primer, pho1′, 5′-GCGATAGGATCCAAGCTTAT-TACTTCATGTTATC-3′ (*Bam*HI), were used. PCR was for eight cycles of 2 min at 94 °C, 1 min 30 s at 42 °C and 2 min 30 s at 72 °C using pho1′ and pho1, pho2 or pho3 as primer pairs, and 1 μg of template DNA as above. After gel purification and digestion with *Nru*I or *Sma*I and *Bam*HI, the fragment was subcloned into pSB/pho. The plasmid was prepared either by *Xma*I digestion followed by mung bean treatment to blunt the 5′ overhang and *Bam*HI digestion (for the *Nru*I/*Bam*HI fragments), or by *Sma*I/*Bam*HI digestion (Figure 1a). The second series was based on the pKK233-2 plasmid. A pair of partially overlapping oligodeoxynucleotides was first used to reconstruct the leader coding sequence of periplasmic Cph [15], while its C-terminal end was modified to adapt it to the consensus (Phe-Ser-Ala-Ser-Ala-Leu-Ala) described earlier [17,20]: 5′-GCGATAACATGTTCA-AAAGCACCCTGGCGGCGATGGCTGCTGTTTTCGC-TCTGTCTG-3′;5′-CGCTATAAGCTTCTGCAG**GCTAGC**-GCGCTCGCGCTGAAAGCAGACAGAGCGAAAACAG-3′. After annealing and filling-in using *Taq* polymerase, the DNA fragment was digested with *Afl*III and *Hin*dIII (underlined) before subcloning between the *Nco*I and *Hin*dIII sites of the pKK233-2 plasmid to generate pKK/cph. The unique *Nhe*I site which was introduced to facilitate later subcloning is shown with bold type. The following primers were then used (the recognition sites for the restriction enzymes noted in parentheses are underlined) to generate the pallidipin coding sequences suited for introduction into pKK/cph: sense primers cph1, 5′-GCGA-TAGCTAGCGGAAGAATGCGAACTCATG-3′ (*Nhe*I) and cph2, 5′-GCGAT AGCTAGCGAAAATCGAAGAAT GCGA-ACTCATG-3′ (*Nhe*I); antisense primer cph1′, 5′-GCGATA-AAGCTTTTATTACTTCATGTTATCACA-3′ (*Hin*dIII). Primers cph1 or cph2 and cph1′ were used to amplify the pallidipin coding sequence by PCR as above. The amplified fragments were purified, digested with *Nhe*I/*Hin*dIII and subcloned into pKK/cph (Figure 1b).

All the constructs were checked by complete DNA sequencing of the inserted fragments using the dideoxy chain-termination method [30] and a sequencing kit with [^{35}S]dATP[S]. Transformation of competent *E. coli* JM105, E15 or BL20 with pallidipin expression constructs or empty plasmids (mock transformation) was carried out using standard methods [31].

Expression and extraction of recombinant pallidipin

An overnight culture of JM105 transformed with the pKK233-2-based cytoplasmic expression construct was diluted 100-fold in LB/ampicillin medium (100 μg/ml) and shaken at 37 °C until it reached an A_{600} of 0.6. Induction was then performed for 6 h at 37 °C in the presence of 1 mM IPTG. Total extracts were prepared from the bacterial pellets after centrifugation. They were analysed by SDS/PAGE and blotting (see below), and by PCR using specific primers to amplify a 276-nucleotide-long fragment corresponding to positions 156 to 431 of the pallidipin cDNA sequence [1]. The resulting products were separated on a 1.5% agarose gel and stained with ethidium bromide [31].

For the pSB/pho plasmids, overnight E15 cultures were diluted to 8% (v/v) in low-phosphate medium and grown for 6 h at 37 °C [26,32,33]. For the pKK/cph plasmids, overnight BL20 cultures were diluted and induced like the JM105 culture above. After induction, the E15 or BL20 bacteria were harvested by centrifugation and resuspended in a one-tenth volume of 50 mM Tris/HCl (pH 8.0)/100 mM NaCl. This preparation was frozen, thawed and centrifuged, thus giving a first supernatant fraction (SN1). The bacterial pellet was resuspended and equilibrated for 10 min at room temperature in 0.5 M saccharose before centrifugation. The supernatant (SN2) was kept for analysis, while the pellet was resuspended in ice-cold water supplemented with 1 mM PMSF and incubated for 10 min on ice. Following this osmotic shock, the cells were centrifuged and the supernatant (SN3) collected. The pellet containing the cytoplasmic fraction (CF) was resuspended in 50 mM Tris/HCl (pH 8.0)/100 mM NaCl.

Purification of recombinant pallidipin

The supernatant fraction containing the bulk of recombinant pallidipin (SN1) was adjusted to pH 4.0 with acetic acid and applied to a cation-exchange column (Mono-S, Pharmacia) using an FPLC system. After washing with a gradient of 0 to 500 mM NaCl in sodium acetate, pH 4, elution of pallidipin was achieved with 20 mM sodium phosphate, pH 7.0. Eluent fractions containing pallidipin, as judged by SDS/PAGE and immunoblotting, were pooled, adjusted to pH 8.0 and applied to an anion-exchange column (Fractogel-EMD-TMAE 650; Merck). Elution of pallidipin was achieved with a gradient of 0 to 1 M NaCl in 20 mM sodium acetate, pH 8.4. For the final purification, eluent fractions containing pallidipin were pooled, concentrated in a Speed-Vac (Bachofer) and subjected to size-exclusion chromatography using Superose 12 (Pharmacia).

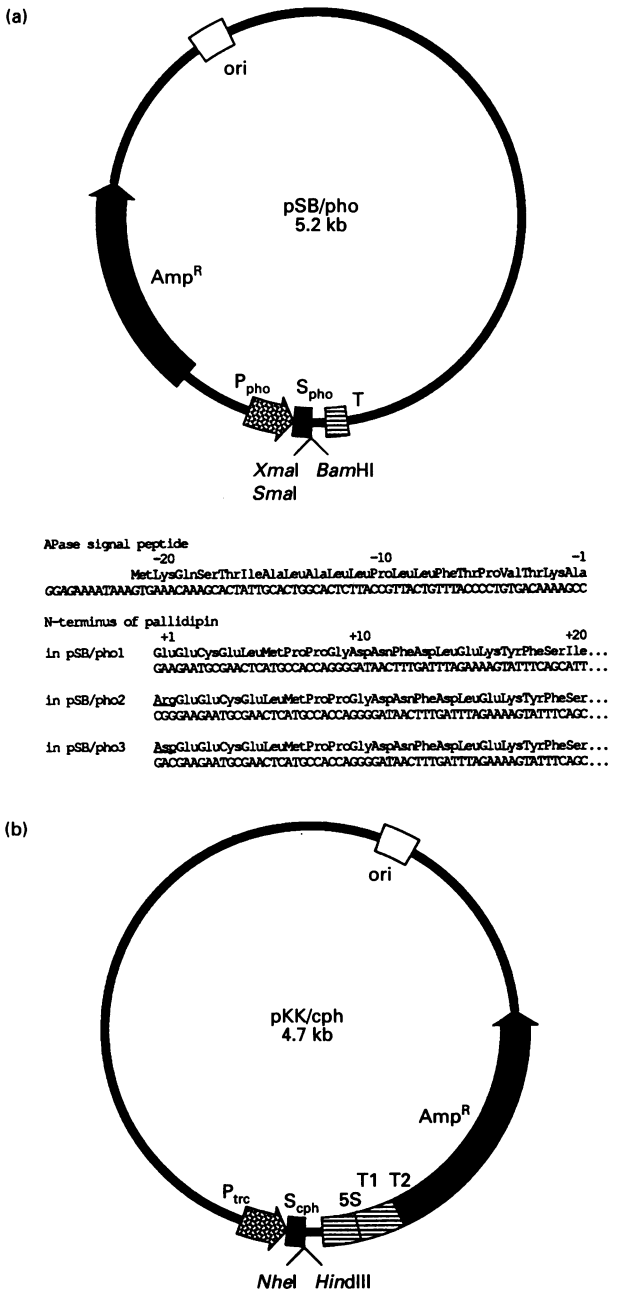

Figure 1 Schematic representation of the pSB/pho (a) and pKK/cph (b)-derived pallidipin expression vectors

The regions corresponding to the promoter (P_{pho} and P_{trc}; spotted), leader peptide (S_{pho} and S_{cph}; diagonally hatched), transcription terminator (T, and 5S and T1T2; horizontally hatched), ampicillin resistance (Amp^R; dark grey) and plasmid origin of replication (ori; open box) are shown. The restriction sites used for the subcloning are indicated. The DNA sequences coding for the leader sequence (preceded by the ribosomal binding site in italics) and for the N-terminal portion of pallidipin are written out with the corresponding amino acid sequences. The additional residues of the variant forms are underlined.

Electrophoresis and protein blotting

SDS/PAGE was performed essentially as described previously [34] using a 5% acrylamide stacking gel and a 12.5% acrylamide separating gel. Gels were fixed and stained with Coomassie Brilliant Blue as described previously [35]. For N-terminal sequencing, the protein was transferred to poly(vinylidene difluoride) sheets (2 h at a constant current of 150 mA). It was then stained essentially as described elsewhere [36], excised and subjected to Edman degradation. For immunodetection, proteins were transferred as above to nitrocellulose BA 85 sheets using 25 mM Tris/192 mM glycine/0.1% SDS/20% methanol as buffer.

Immunostaining of protein blots

The protein blots were blocked with non-fat dry milk (5% in PBS/0.05% Tween 80 for 60 min at 25 °C), incubated for 90 min at 25 °C with an antiserum specific for pallidipin (diluted 1:1000 in PBS/0.05% Tween 80) [1], and washed three times with PBS/0.05% Tween 80. This was followed by a 90-min-long incubation with anti-(rabbit IgG)-conjugated APase (diluted 1:2000 in PBS/0.05% Tween 80) at 25 °C. After washing as above, the staining was initiated by addition of Nitro Blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, as described previously [37].

Amino acid sequencing

Edman degradation was used for the N-terminal sequence analysis of recombinant pallidipin variants, using a model 477A protein sequencer with an on-line 120A phenylthiohydantoin amino acid analyser (Applied Biosystems).

Platelet aggregation assay

The assay was carried out essentially as described previously [1]. Briefly, human blood was collected into a one-sixth volume of 71 mM citric acid/85 mM trisodium citrate/111 mM glucose. Platelet-rich plasma was obtained by centrifugation at 135 *g* for 20 min. Pallidipin (400 nM) was incubated with 500 μl of platelet-rich plasma for 1 min at 37 °C before the addition of collagen (2 μg/ml). The aggregation was monitored using a Micron aggregometer and the maximum value was determined. For the dose–response analysis, 20 to 250 nM pallidipin was used.

RESULTS

Expression of recombinant pallidipin

The objective of this work was the construction of high-level *E. coli* expression plasmids for pallidipin, if possible with an N-terminus identical to that of the natural form. Initial experiments aiming at cytoplasmic expression and where the coding sequence of pallidipin, without its signal peptide coding part, was placed downstream of the *trc* promoter in the pKK233-2 plasmid, did not allow the detection of recombinant protein (Figures 2a and 2b). The presence of pallidipin coding sequences at the time of induction and of harvesting was confirmed by PCR (Figure 2c).

Expression plasmids with the pallidipin coding sequence placed downstream of the DNA sequence for the leader peptide of the two abundant periplasmic *E. coli* proteins APase and Cph, were therefore constructed. Three pSB/pho-derived plasmids were made (Figure 1a). In pSB/pho-1, the region coding for mature pallidipin was fused directly to the APase leader peptide coding

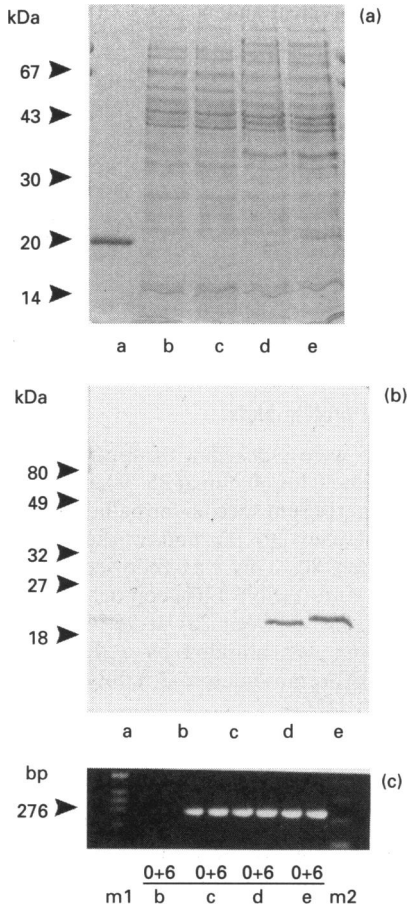

Figure 2 Comparison between the cytoplasmic and periplasmic expression systems

E. coli was transformed either with a control pKK233-2 plasmid (lanes b), with the cytoplasmic expression construct (lanes c), with pKK/cph-1 (lanes d) or with pKK/cph-2 (lanes e) and induced before preparing total extracts as described in the Experimental section. Equivalent aliquots were analysed by SDS/PAGE as well as pallidipin purified from saliva (lanes a). The proteins were either stained with Coomassie Brilliant Blue (**a**) or blotted on to nitrocellulose membranes and developed using pallidipin-specific antibodies (**b**). PCR was used to amplify a pallidipin-coding sequence fragment from samples taken at the beginning (0) and end (+6) of the induction period. The 1 kb (m1) and 123 bp (m2) DNA molecular mass markers (Gibco BRL) were loaded in parallel (**c**).

part, thereby creating the Thr-Lys-Ala ↓ Glu cleavage site. Since mature APase starts with the basic Arg residue, a second plasmid, pSB/pho-2, where the corresponding codon was intercalated, was also constructed (cleavage site: Thr-Lys-Ala ↓ Arg-Glu). However, in view of the remarkably acidic character of the N-terminus of mature pallidipin, it seemed reasonable to assume that this feature might indeed be of importance for the biological properties of the protein. With this in mind we tested an additional construct where an Asp residue had been introduced (pSB/pho-3; cleavage site: Thr-Lys-Ala ↓ Asp-Glu).

For the pKK/cph expression vectors, the coding region for the modified leader peptide of periplasmic Cph was first subcloned into the pKK233-2 plasmid, downstream of the *trc* promoter (Figure 1b). The pallidipin cDNA was then introduced to create a direct fusion between both coding sequences (pKK/cph-1; Ala-Leu-Ala ↓ Glu). In pKK/cph-2, the ideal leader peptidase cleavage site [20] was recreated by adding two amino acids to the N-terminus of pallidipin (Ala-Leu-Ala ↓ Lys-Ile-Glu).

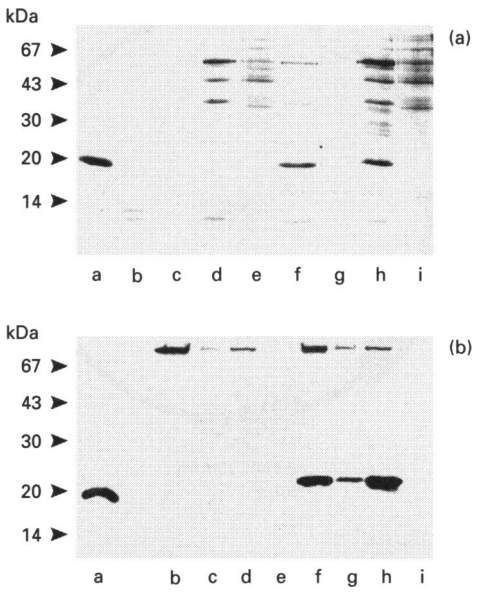

Figure 3 Stepwise extraction of pallidipin from *E. coli*

E. coli was transformed either with a control pSB95 plasmid (lanes b–e) or with the pSB/pho-3 plasmid (lanes f–i), induced, and then extracted as described in the Experimental section. Aliquots of the fractions were separated by SDS/PAGE and then either stained (**a**) or blotted on to nitrocellulose membranes and developed using pallidipin-specific antibodies (**b**). Lane a, pallidipin purified from saliva; lanes b and f, SN1; lanes c and g, SN2; lanes d and h, SN3; lanes e and i, CF.

The expression plasmids were introduced into the E15 (pSB/pho) or BL20 (pKK/cph) bacterial strains. In all instances, induction with low-phosphate buffer or IPTG led to the over-expression of a protein of approximately 19 kDa, as observed on SDS/PAGE (see below).

Compartmentalization of expressed pallidipin

Stepwise extraction from bacterial fractions resulted in three SN fractions and one bacterial pellet designated as CF. These fractions were analysed by SDS/PAGE and immunoblotting. A typical extraction of aspartyl-pallidipin is shown in Figure 3. Pallidipin was mainly found in SN1 and SN3, i.e. in the supernatants obtained after the freeze–thaw step and the osmotic shock respectively (Figure 3a). On immunoblots using specific antibodies raised against pallidipin these bands were intensely stained, showing that they indeed corresponded to pallidipin (Figure 3b). When the freeze–thaw step was omitted and thus no SN1 obtained, pallidipin was found exclusively in the osmotic shock (SN3) fraction (results not shown). It was therefore concluded that pallidipin accumulated in the periplasmic space, from which it was released by freezing and thawing. Since it showed a significantly higher ratio of pallidipin versus contaminating bacterial proteins, the SN1 fraction was used for the subsequent purification, despite the lower overall yield.

Purification of recombinant pallidipin

For a careful comparison of the *E.coli*-expressed pallidipin forms and the natural protein purified from *T. pallidipennis* saliva, a procedure was developed to purify all variants from the SN1 fraction. A platelet aggregation assay was used to monitor the purification process. To remove a non-specific bacterial inhibitory

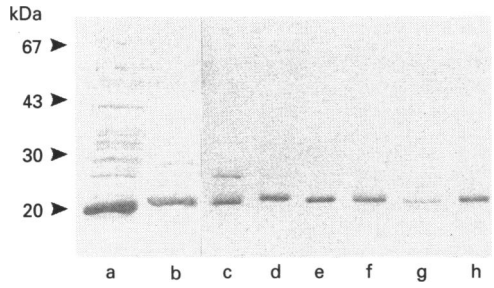

Figure 4 Purification of pallidipin variants

The pallidipin variants were purified from SN1 by chromatography on a Mono-S cation-exchange column (lane a), followed by Fractogel EMD-TMAE 650 (lane b), and Superose 12 (lanes c–h). Lane c, pallidipin from pSB/pho-1; lane d, arginyl-pallidipin from pSB/pho-2; lane e, aspartyl-pallidipin from pSB/pho-3; lane f, pallidipin from pKK/cph-1; lane g, lysyl-isoleucyl-pallidipin from pKK/cph-2; lane h, native pallidipin from the saliva of *T. pallidipennis*.

Table 1 Purification yields for the recombinant pallidipin forms and biological activity

Recombinant pallidipin was purified from one litre of induced bacterial culture with the three-step protocol described in the Experimental section. The inhibitory activity (IC_{50}) was assessed in a platelet aggregation assay using 20 to 250 nM pallidipin. Native pallidipin was purified from the saliva of *T. pallidipennis* as described earlier [1]. The additional residues of the variant forms are underlined. Abbreviations: Re, recombinant; n.a., not applicable.

Form	N-terminus	Construct	Yield (μg)	IC_{50} (nM)
Re pallidipin	EECE...	pSB/pho-1	461	33
Re arginyl-pallidipin	REECE...	pSB/pho-2	298	44
Re aspartyl-pallidipin	DEECE...	pSB/pho-3	864	33
Re pallidipin	EECE...	pKK/cph-1	156	89
Re lysyl-isoleucyl-pallidipin	KIEECE...	pKK/cph-2	80	> 778
Native pallidipin	EECE...	n.a.	n.a.	89

activity, cation-exchange chromatography on Mono-S was introduced as an initial purification step. The SN1 fractions from the different transformants, but not from the control, were all found to strongly inhibit collagen-induced platelet aggregation (results not shown).

Further purification was achieved by anion-exchange chromatography on Fractogel EMD-TMAE followed by size-exclusion chromatography on Superose 12. Highly purified recombinant pallidipin corresponding to all the expression vectors we engineered was obtained (Figure 4). N-terminal amino acid sequencing showed that in four out of five instances pallidipin had been processed at the expected site. In the case of the pSB/pho-2 construct, the N-terminal amino acid proved difficult to identify unequivocally; however, the subsequent residues were as expected.

The yields ranged from 80 to 864 μg starting from one litre of culture broth (Table 1). The three pSB/pho-derived constructs gave the highest levels. However, significant differences were observed within the expression systems: more purified material was obtained when the N-terminal residue of pallidipin was acidic (as in the wild-type form or in the Asp variant), whereas lower yields resulted from a basic N-terminus (as in the Arg and Lys-Ile variants).

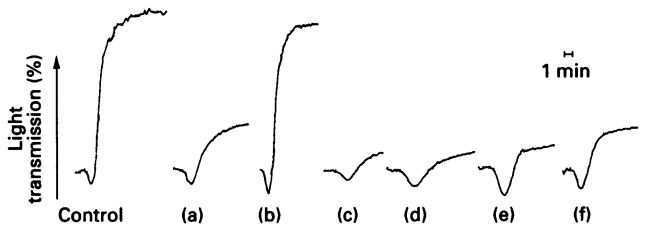

Figure 5 Effect of recombinant pallidipin on collagen-induced platelet aggregation

The inhibitory effects of the recombinant variant forms derived from pSB/cph-1 (**a**), -2 (**b**), pKK/pho-1 (**c**), -2 (**d**) and -3 (**e**) and of native pallidipin (**f**) were compared. The assay was carried out with 400 nM pallidipin. In the control reaction no pallidipin was added.

Biological properties of recombinant pallidipin

The biological activity of the different recombinant pallidipin forms purified from the periplasmic space of *E. coli* was determined. Platelet-rich plasma was incubated with pallidipin (400 nM) and aggregation was induced by adding collagen. Native pallidipin was used as a positive control. We found the inhibitory effect of the various *E. coli*-derived forms to be equivalent to that of *T. pallidipennis* salivary pallidipin, with the exception of lysyl-isoleucyl-pallidipin, i.e. the product of the pKK/cph-2 construct, which exhibited a clearly reduced activity (Figure 5). The IC_{50} values of the different forms were calculated from the dose–response curves of the variants, using concentrations varying from 20 to 250 nM. It showed the IC_{50} of lysyl-isoleucyl-pallidipin to be more than ten times higher than that of the other forms (Table 1).

DISCUSSION

We have achieved high-level synthesis of pallidipin by transformed *E. coli* cells and export of the protein into the periplasm. All three pSB/pho-derived constructs gave better yields than those based on pKK/cph. This could be due to the different expression vectors or bacterial strains used. The combination of the bacterial APase promoter and leader peptide has previously been used with varying results. Accurate processing and yields comparable with ours were reported for periplasmic expression of human growth hormone in *E. coli* [4]. Very high expression levels were reported for the 22 kDa N-terminal end of human apolipoprotein E, although when the whole 35 kDa protein was expressed, production was reduced [6]. A lower growth temperature (30 °C) and the use of a bacterial strain lacking the endogenous APase gene (*phoA*) were found to improve the results. One problem encountered was the deletion of the initial amino acid in the mature product, due to improper processing or proteolytic cleavage in the periplasm. Conversely, attempts to produce mature human interleukin-1β or interferon-α2c in the periplasm of *E. coli* were altogether unsuccessful when employing the APase leader peptide [5,23,38]. In both cases the use of another leader peptide (from the outer membrane protein or the heat-stable enterotoxin II of *E. coli* respectively) solved the problem.

The use of the leader peptide from bacterial periplasmic Cph for export of heterologous proteins has so far not been reported. Our studies show that it can be used successfully, even though less efficiently than the APase leader peptide. Expression of pallidipin in the BL20 strain, known to be deficient in periplasmic proteases and therefore likely to improve the expression

[28,29,39], was still not sufficient to reach the levels obtained with the pSB/pho-based vectors. Further experimentation will be necessary to find out whether the use of promoters other than *trc*, which we tested in combination with the Cph leader sequence, will allow higher expression.

Altogether these results show that not all combinations of bacterial leader peptides with a given eukaryotic protein will allow equivalent translocation efficiency into the periplasm, pointing not only to the importance of the cleavage site but also to the influence of the leader peptide on the downstream protein. It has, for instance, been suggested that the leader sequence retards tertiary structure formation and thereby favours translocation [40,41]. Export and processing of a heterologous protein in *E. coli* is at present still not fully predictable, reflecting our only partial knowledge of the chain of events taking place [41]. Attempts to overexpress the bacterial leader peptidase [5] or the chaperone SecB supposed to improve exportability [38] have so far not led to substantial improvements.

The presence of an extra lysyl-isoleucyl dipeptide at the N-terminus of pallidipin dramatically affected its specific inhibitory activity in the platelet aggregation test. Conversely, none of the other variants significantly differed from the wild-type form in this respect. It is not known at present whether the N-terminus of the protein plays an important functional role, but its marked acidic character is noteworthy. We found that the additional presence of one basic residue as in the Arg form does not interfere with the activity, whereas the Lys-Ile form is at least ten times less active. This is difficult to interpret in view of our limited information on the mechanism of action of pallidipin and especially on the regions of the protein which are involved in it. The availability of substantial amounts of recombinant pallidipin will now allow us to investigate the mode of action at the molecular level and to test the efficacy in animal models of this anti-thrombotic agent.

We thank Dr. Werner Boidol for the gift of the pSB/pho plasmid and of the E15 strain, and Dr. Irene Kuhn for the BL20 strain. We gratefully acknowledge the efficient help of Carola Schlegel with the generation of expression plasmids and the expertise of Liane Dummann, Silke Finster, Uwe Jerke and Anja Wegg in the purification and characterization of recombinant proteins. We are indebted to Daniela Sauvageot for oligodeoxynucleotide synthesis.

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Received 1 September 1994/7 December 1994; accepted 9 December 1994