Introduction of Guest Peptides into *Escherichia coli* Alkaline Phosphatase

EXCISION AND PURIFICATION OF A DYNORPHIN ANALOGUE FROM AN ACTIVE CHIMERIC PROTEIN*

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High relative mutability may be a common property of the surfaces of all or most proteins and may be exploited during evolution not only to alter molecular recognition but to modify catalytic functions as well. Conservative amino acid substitutions often can be expected to cause minimal structural alterations, but the properties of protein surfaces and the mechanisms of protein folding that accommodate length variation without loss of function are not understood. To begin to address these aspects of protein structure and folding, we have constructed short amino acid insertions in the *Escherichia coli* alkaline phosphatase polypeptide by linker insertion mutagenesis of the *phoA* gene and have examined correlations between mutant protein function and position of the insertions relative to the x-ray map of wild type alkaline phosphatase determined by Wycoff and colleagues (Sowadski, J. M., Foster, B. A., and Wycoff, H. W. (1981) J. Mol. Biol. 150, 245–272). Mutant protein enzymatic function was generally tolerant of insertions in exterior loops, but was inactivated by insertion within α-helical or β-strand structural elements. We further demonstrate that these tolerant surface loops can serve as vehicles for high level expression and stabilization of larger foreign peptide sequences, using a 15-residue analogue of dynorphin as an example. Insertion of the dynorphin “guest” peptide probably caused only a local structural perturbation of the alkaline phosphatase carrier since the hybrid protein retained enzymatic activity, was exported efficiently to the periplasmic space, and could be purified by anion-exchange chromatography using a protocol developed for alkaline phosphatase itself. The guest peptide was recovered from one of these fusion proteins intact and in high yield by protease digestion in vitro and was then purified by cation-exchange chromatography to near homogeneity in a single step.

Variations in polypeptide chain length occur during evolution of protein structure and function and can also be generated rapidly, for example, in antibodies by recombination events in the assembly of immunoglobulin genes (reviewed in Refs. 1 and 2). At the level of protein tertiary structure, these insertions or deletions of sequence usually occur within loops on the protein surface and are tolerated by the rest of the protein structure, which remains essentially unaltered. Variable loops on the surface of an invariant protein scaffold are a vital aspect of certain molecular recognition processes. In the case of antibodies, the expansion or contraction of loops alters the shape of the antigen binding site and therefore contributes to the diversity of the immune response. Antigenic determinants themselves may be contained within loops that also expand or shrink, thus altering the antigenic properties of the protein. For example, a major neutralizing immunogenic site on the VP1 capsomer of foot and mouth disease virus corresponds to a loop which is absent from the VP1 capsomers of the related picornaviruses poliovirus and rhinovirus (3, 4).

Whether tolerance of length variation in surface loops is a general property of protein structure or is restricted to a subset of proteins whose functions require changes in shape such as antibodies, antigens, or allosteric enzymes is unknown. Conservation of polypeptide chain length of triose-phosphate isomerase, for example, has been observed in evolution from bacteria to mammals (5). To explore the structural basis of length variation in a model system, we used site-directed mutagenesis to construct di- or tripeptide insertions in the *Escherichia coli* AP1 polypeptide at locations that corresponded to loops, α-helices, or β-strands in the wild type tertiary structure defined by Wycoff and colleagues (6). In accord with our expectations, we show that enzymatic activity was fully or partially retained when the insertions were targeted to loops, but was abolished when an α-helix or β-strand was interrupted. We then decided to exploit the tolerant sites further, in order to design an expression vehicle for a 15-residue analogue of the opioid peptide dynorphin A (7). This approach was expected to have several advantages over most other expression vehicles for small peptides, which consist of hybrid structures with the desired peptide attached to the carboxyl-terminal end of the larger protein (8, 9). By inserting the smaller peptide into the middle of the carrier polypeptide chain, it is protected from exopeptidase digestion, and endopeptidase digestion should be readily detectable by monitoring the alkaline phosphatase subunit molecular weight by electrophoresis in denaturing gels. Also, tolerance of the insertion by the rest of the enzyme structure should allow the hybrid protein to be recovered by the standard methods used for the native enzyme. The results we present demonstrate that this approach is useful for preparation of the dynorphin analogue and might be applied to the rational design of many other carrier protein-peptide systems.

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2 The abbreviations used are: AP, the *E. coli* alkaline phosphatase; SDS, sodium dodecyl sulfate; GPI, guinea pig ileum; V8, protease from *Staphylococcus aureus*; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinoethane sulfonic acid.
Materials and methods

bacterial and genetic procedures—The phoA gene used in these studies was isolated from the E. coli chromosome by Inouye et al. (10). A 2.5-kilobase HindIII-BamHI restriction fragment containing the phoA gene and its flanking regions was inserted into pBR322. To release the effect size of phoA during mutagenesis, pBR322 DNA between the AccI sites at nucleotides 651 and 2246 was deleted. Linker insertion mutagenesis of this DNA was performed as described previously (11).

Briefly, supercoiled plasmid DNA was partially digested with the restriction enzymes shown in Table I and electrophoresed in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) at 2.5 V/cm for 30 min in 0.6% low melting point agarose gels. Gel slices containing the full-length linear species were mixed with an equal volume of TEN buffer (10 mM Tris, 1 mM EDTA, 250 mM NaCl, pH 8), incubated at 68 °C for 15 min, extracted twice with phenol and once with chloroform, and precipitated with 2 volumes of ethanol at −20 °C overnight. DNAs were collected by centrifugation, washed once with 1 ml of 70% ethanol, and ligated to a 5-fold molar excess of phosphorylated Smal DNA linkers (CCCGGG, New England Biolabs) in ligation buffer (50 mM Tris, pH 7.8, 10 mM MgCl₂, 20 mM dithiothreitol, 1 mM ATP) containing 5 units of T4 ligase for 2 h at 15 °C. Mid-log phase cultures of the AP indicator strain AW1043 (10) made competent by the method of Cohen and Miller (12) were transformed to ampicillin resistance by electroporation. Transformants were selected in LB broth containing 150 μg/liter penicillin G (Sigma), and plasmid DNA prepared by alkaline lysis (13) of these populations was digested with Smal and electrophoresed as above to separate mutant DNAs (which contain Smal linkers and therefore are linearized) from the background of circular, Smal-resistant wild type DNA. Linear mutant DNAs were extracted from the gels, recircularized by T4 ligase, and transfected into AW1043. Smal linker insertions in plasmid DNA from single colony isolates were mapped by restriction enzyme digestion and confirmed by local DNA sequencing (14). The results of the mutagenesis are shown in Table I. The method of Brickman and Beckwith (15) was adapted to measure AP enzymatic activity in situ. The absorbance of a growing culture was measured at 600 nm, and 0.01-0.01 OD units were added to 0.9 ml of 1 M Tris-HCl, pH 8.0, containing 2 drops of chloroform and 1 drop of 0.1% SDS. The suspension was mixed by shaking to permeabilize the cells, and 20 μl of p-nitrophenyl phosphate (Sigma 104, 3 mg/ml) was added. The absorbance at 410 nm was recorded against time.

construction of AP-dynorphin HybriD—An oligonucleotide encoding a dynorphin analogue and its complementary strand (Fig. I) were synthesized using β-cyanoethyl phosphoramidite chemistry (16) on a Synquest Microsyn 1450A DNA synthesizer. Oligonucleotides were purified by electrophoresis in 6% polyacrylamide gels containing 7 M urea following the method of Maxam and Gilbert (17). Equimolar amounts of each strand (50-100 pmol) were dissolved in 10 μl of H₂O, heated to 95 °C, cooled slowly for annealing. The annealed strands (40 pmol) were phosphorylated in 20 μl of buffer (60 mM Tris pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM ATP) containing 5 units of polynucleotide kinase for 30 min at 37 °C. A portion of this reaction (1 μl, 2 pmol of strands) was added to 19 μl of ligation buffer containing 0.2 pmol of Smal-digested pPF1038 or pPF1041 (Table I) and 1 unit of T4 DNA ligase, and it was incubated for 2 h at 15 °C. Competent AW1043 cells were transformed to ampicillin resistance by the ligated DNA. The structure and location of the dynorphin oligonucleotide insertion were confirmed by local DNA sequencing.

isolation and purification of AP-dynorphin Hybrid Protein—The OmpF protein-deficient E. coli strain BL21 (17) was transformed by pPF1038 (ompPF1038 containing dynorphin oligonucleotide insertion) and grown to stationary phase overnight in N broth (10 g/liter baetryptosine, 1 g/liter yeast extract, 1 g/liter dextrose, 8 g/liter NaCl) and diluted 1:10 into MOPS minimal medium (18) supplemented with 0.1 mM KH₂PO₄, 4 g/liter dextrose, 1.5 g/liter casamino acids, and 50 mg/liter penicillin G to induce the synthesis of the AP-dynorphin hybrid. After shaking at 15 °C for 5 h, the cells were collected by centrifugation, washed with 100 mM Tris, pH 8 (20 ml/liter), and resuspended in 20% (w/v) sucrose, 50 mM Tris, pH 8, to a final volume of 10 ml/liter of culture. Lysozyme was added to 30 μg/ml, and the cell suspension was frozen at −70 °C and thawed at 37 °C for 20 min. The suspension was then incubated at room temperature for 10 min in the presence of 10 mM MgSO₄, and stored frozen at −20 °C. The dynorphin peptide analogue was recovered by digestion of this material with V8 protease (Worthington) using 1 unit/5 μg of hybrid at 37 °C for 1 h. Proteolysis was terminated by adjusting the reaction to 5 mM EDTA and boiling for 15 min.

Guinea Pig ileum assays—Whole segments of guinea pig ileum were mounted between platinum electrodes in an organ bath containing modified Krebs-Ringer at 37 °C and subjected to square wave voltage pulses, as described previously (19). The electrically stimulated muscle contraction tensions were allowed to equilibrate to a constant level over a period of 30-60 min, during which time the Ringer solution was exchanged several times, and then the muscle was challenged with chromatographic fractions. When opioid activity causing inhibition of the electrically stimulated contractions was detected, this was confirmed by addition of the opioid antagonist naloxone (10 μM) to the bathing solution and observing complete reversibility of the inhibition. Muscle sample isolated in this fashion could be employed over a period of several hours in order to screen multiple chromatographic fractions reliably and reproducibly, provided that the bathing Ringer solution was exchanged frequently and the muscles were washed extensively with the Ringer after each naloxone addition.

high pressure liquid chromatography—HPLC was performed using a Beckman system consisting of two model 114M solvent pumps operating under the control of a model 421A controller and a model 164 detector. Samples were eluted from a Vydac C₈ (5 μm, 300 Å) analytical column using acetonitrile gradients in 0.005% (v/v) aqueous trifluoroacetic acid and a flow rate of 1.2 ml/min.

results

linker insertion mutagenesis—to examine the tolerance of E. coli alkaline phosphatase (AP) to amino acid insertions, we subjected the phoA gene to linker insertion mutagenesis. Circular plasmid DNA containing the wild type phoA gene (10) was linearized at restriction enzyme sites within phoA and recircularized by T4 DNA ligase in the presence of excess Smal linkers. Insertion of a linker introduced a Smal restriction site that was unique in both phoA and plasmid vector sequences, preserved the AP translational reading frame and resulted in the insertion of two or three novel amino acids in the AP primary structure. The mutations were characterized by restriction enzyme mapping and local DNA sequencing, and their effects on AP function were assessed by expression of mutant genes in E. coli strain AW1043, which carries a deletion in the chromosomal phoA gene (10). Cells from induced cultures were assayed for AP activity in situ (15), and whole cell lysates or periplasm were electrophoresed in SDS-containing polyacrylamide gels to monitor accumulation and localization of mutant gene products. The results of these analyses and the locations of the insertions relative to the x-ray map of wild type AP (1) are shown in Table I. Dipetide insertions (Pro-Gly) within β-strand F or α-helix 336-358 abolished AP activity, yet these proteins were exported to the periplasm and accumulated to wild type levels (data not shown). All other insertions were located in external loops in the AP tertiary structure. With the exception of PF1060, these mutants retained activity and accumulated in the periplasm to high levels (not shown). The levels of PF1060 AP protein were low, which was surprising since this region of AP is nonessential for activity and can be deleted or substituted by large fragments of heterologous proteins (20, 21). Inspection of the DNA sequence revealed a GC-rich region adjacent and complementary to the linker insertion mutation. Whether potential DNA or RNA secondary structures here
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Table I
Characterization of alkaline phosphatase insertion mutants

| Plasmid       | Restriction site modified | Mutant sequence* | Location in wild type tertiary structure* | Wild type activity per cell | Concentration in periplasm |%
|---------------|---------------------------|------------------|-------------------------------------------|-----------------------------|----------------------------|
| pPF1060       | Ddel*                     | Ala His Pro Gly Gin | Loop                                      | 3                          | Low                         |
| pPF1040       | DpnI                      | Asp Pro Gly His   | Loop                                      | 24                         | High                        |
| pPF1038       | FnuDII                    | Ser Pro Gly Arg   | Loop                                      | 84                         | High                        |
| pPF1041       | DpnI                      | Gly Pro Gly Ser   | Loop                                      | 102                        | High                        |
| pPF1037       | HaeIII                    | Gly Pro Gly Gin   | β-Strand                                  | <0.01                      | High                        |
| pPF1036       | RanI                      | Val Pro Gly Gin   | α-helix                                   | <0.01                      | High                        |
| pPF1054       | AluI                      | Lys Ala Arg Ala   | Loop                                      | 71                          | Variable                    |

* Amino acids are numbered relative to the sequence of mature alkaline phosphatase (33). Inserted residues are italicized, and inserted nucleotides are underlined in lower case.

† The tertiary structure of wild type AP was determined by Wycoff and colleagues (6).

‡ Determined by measuring activity in situ as described under "Materials and Methods."

§ Periplasm was prepared by lysozyme-EDTA treatment of cells. AP concentration in different mutants was compared by electrophoresis of periplasm samples in SDS-containing polyacrylamide gels.

¶ The recessed 3' ends resulting from Ddel digestion were filled in by DNA synthesis using the Klenow fragment of DNA polymerase I before ligation to SmaI linkers. The original Ddel site, CTCAG, is converted to CTCAC(ccccggg/TCAG (SmaI linker in parenthesis).

†† PF1064 cells form filaments and lose viability if the culture is efficiently aerated during mutant phoA gene expression. The reported activity was measured in cells from a poorly aerated culture (low surface area/volume ratio in a tube with an air-tight cap).

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Fig. 1. Design of an oligonucleotide encoding a dynorphin analogue flanked by V8 protease cleavage sites. The oligonucleotide shown and its complementary strand were synthesized, annealed, and ligated to SmaI-digested pPF1038 or pPF1041 as described in text. Peptide bonds hydrolyzed by V8 protease are indicated by arrows. Also shown are restriction sites that were included to determine the orientation and DNA sequence of the oligonucleotide after its insertion in the expression vectors.

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interfere with expression of the mutant AP has not been examined.

Construction of AP-Dynorphin Fusion Proteins—To examine further the variability of the AP surface and its potential utility, the loops defined by the mutations of pPF1038 and pPF1041 were selected as sites for the insertion of an analogue of the opioid peptide dynorphin (7). This amino acid sequence (Fig. 1) is highly charged and, therefore, compatible with a surface location. The inserted amino acid sequence included flanking glutamic acid residues so that the dynorphin analogue could be excised from the AP carrier by digestion with V8 protease (22). Synthetic oligonucleotides encoding this insertion (Fig. 1) were ligated into the SmaI sites of pPF1038 and pPF1041, and the resulting DNAs were used to transform AW1043 to ampicillin resistance. Both strains formed blue colonies on AP indicator medium (15), indicating that the AP-dynorphin hybrid proteins were enzymatically active. The expression and isolation of both hybrid proteins were analyzed by SDS-polyacrylamide gel electrophoresis and found to be similar. Fig. 2 shows the results obtained with the pPF1041 derivative (PF1160), that contains the dynorphin analogue inserted between residues 190–191 of the wild type alkaline phosphatase sequence. Cells from a late-log phase culture of AW1043(pPF1160) were lysed by boiling in SDS-containing sample buffer, and the lysate was loaded in lane 3. The abundant doublet of apparent molecular mass 52–54 kDa was not present in lysates of AW1043 and could be repressed in AW1043(pPF1160) cells by growth in media supplemented with 10 mM KH2PO4 (data not shown). The doublet probably corresponds to the precursor and mature forms of alkaline phosphatase, and we have observed this pattern in all strains that contain wild type or mutant phoA genes on high copy number plasmids. Late-log phase cells of AW1043(pPF1160) were treated with lysozyme and EDTA to release the contents of the periplasmic space which were loaded in lane 4. The expected 52 kDa product was not observed, but we found two abundant, equimolar bands of 22.9 and 28.2 kDa. We concluded that these two bands correspond to the AP hybrid protein that had been nicked proteolytically within or near to the guest peptide insert, based on the predicted molecular weights of the fragments that would be produced by such a proteolytic event, their equimolarity, and their copurification with AP activity by anion exchange chromatography (data not shown). Similar proteolytic nicking of T7 RNA polymerase by the OmpT protease of E. coli has been reported (17). To examine whether the OmpT protease also cleaved the AP-
FIG. 2. Expression and isolation of AP-dynorphin hybrid proteins. Stationary phase cultures of AW1043(pPF1160) or BL21(pPF1160) were diluted into MOPS minimal medium (18) supplemented with 0.1 mM KH₂PO₄ to induce phoA expression. When cultures reached late log phase, a 1-ml sample was centrifuged, and the cell pellet was lysed by boiling in 0.1 ml of sample buffer (20 mM Tris-HCl, pH 6.8, 6% w/v SDS, 14% w/v 2-mercaptoethanol, 28% w/v glycerol, 0.01% w/v bromphenol blue). Lane 1 contains 5 µl of BL21(pPF1160) lysate; lane 3 contains 5 µl of AW1043(pPF1160) lysate. The remaining culture was treated with lysozyme and EDTA to release the contents of the periplasmic space. Lane 2, BL21 (pPF1160) periplasm; lane 4, AW1043(pPF1160) periplasm. Lane 5 contains the following molecular mass standards: bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa. Lane 6 contains purified wild type AP subunit.

dynorphin hybrid proteins an OmpT-deficient strain, BL21 (17), was transformed by pPF1160. Cells from a late-log phase culture of BL21(pPF1160) were lysed by boiling in sample buffer and the lysate loaded in lane 1 (Fig. 2). As with AW1043(pPF1160), an abundant doublet of 52-54 kDa was observed. When periplasm from BL21 (pPF1160) was analyzed we found an abundant 52-kDa protein (lane 2) and no proteins that comigrated with the 22.9- and 28.2-kDa proteins discussed above. This 52-kDa protein copurified with AP activity on anion exchange chromatography and could be converted to stable fragments of 22.9 and 28.2 kDa by incubating with purified outer membranes of AW1043 cells (data not shown), indicating that OmpT does nick the AP-dynorphin hybrid. Typically 2 mg of hybrid protein purified by anion-exchange chromatography was obtained from 2.5 g wet weight of BL21(pPF1160) cells (1.0 liter of culture).

Excision and Purification of Dynorphin from the AP Carrier—The AP-dynorphin hybrid protein was purified by anion-exchange chromatography of BL21 (pPF1160) periplasm and dialyzed against 50 mM phosphate buffer, pH 7.8. To recover the dynorphin analogue peptide, fusion proteins were digested with V8 protease (22) and chromatographed by reversed-phase HPLC. Fig. 3 shows a portion of a representative HPLC trace of the V8 digest. Major peaks were collected separately and tested for activity in the GPI assay. The fraction corresponding to the A₂₁₃₅₇₉ peak eluting at 35 min (23% v/v acetonitrile) inhibited 90% of the electrically stimulated muscle contractions in a naloxone-reversible fashion, when tested in this way (see Fig. 4). No other fractions displayed significant inhibitory activity.

Cation-exchange chromatography was evaluated as an alternative purification strategy. The V8 digest was applied to a column of SP-Sephadex C25, and the bound peptides were eluted with a gradient of NaCl (Fig. 5). Dynorphin activity was predominantly in the most tightly bound fraction, as expected for a highly basic peptide. Fractions containing dynorphin activity were pooled as indicated in Fig. 5, and analyzed by reversed-phase HPLC. In contrast to the complex trace shown in Fig. 3, the HPLC trace of the SP-Sephadex pool indicated only two components, a major one containing dynorphin-like GPI activity and eluting at 24% acetonitrile, followed by a much smaller, near-baseline separated compo-
Unexpected amino acids were observed in any degradation cycle and a 16th cycle indicated correctly that the peptide 24. Apart from small quantities of alanine (25 pmol), glycine Arg, 220; Pro, 133; Lys, 107; Leu, 100; Lys, 109; Leu, 73; Glu, 248; Gly, 279; Phe, 329; Leu, 376; Arg, 221; Arg, 262; Ile, 256; phenylthiohydantoin derivatives in each cycle: Tyr, 344; Gly, with the following yields (picomoles) of the amino acid Phe, 0.9 (1); Pro, 1.3 (1); Tyr, 0.9 (1). Sequence analysis by automated Edman degradation gave the expected sequence quantity of phenol, amino acid analysis gave: Arg, 3.1 (3); Pro, 1.0; Tyr, 0.9 (1). The surface of the target protein may constrain the conformation of the inserted "guest" peptide. This is evident in hybrids where the carrier protein function has been retained, which implies that carrier structure has also been largely preserved. In such cases, the insertions may resemble the Greek letter Q or with the amino and carboxyl termini juxtaposed by the carrier protein surface and the remainder looped out into solvent. The Q-like structures of this type have been proposed as a new class of compact protein secondary structure (31). Most the active hybrids that we have constructed are stable to proteolysis in situ, supporting a model that the inserted sequences adopt protease-resistant, compact conformations. Hofnung and colleagues also report the construction of stable hybrids of the E. coli LamB protein that contain sequential antigenic determinants of viral polypeptides as guest peptides. The sensitivity of the AP-dynorphin hybrid to cleavage by the OmpT protease within or close to the site of the guest peptide insertion, which is only observed after cell lysis, may result from the high content of basic amino acids within this guest peptide and its possible disordered conformation on the carrier protein surface. It is interesting that OmpT does not cleave elsewhere in the hybrid, suggesting that the carrier structure is similar to the protease resistant wild type AP structure. The structural integrity of the carrier is further demonstrated by its partial retention of enzymatic activity.

The dynorphin analogue was recovered from the hybrid AP-dynorphin construct in high overall yield and in an intact and highly purified form, after only two low-resolution chromatographic steps from the cell lysate (Fig. 6). This demonstrates that the tolerance of surface regions of AP for insertion mutagenesis may be exploited to great advantage in the preparation of small peptides through gene expression in vivo. Previous attempts to achieve similar goals have usually involved attachment of small peptides to the carboxyl terminus of a carrier protein via an amino acid linkage chosen to allow subsequent cleavage at that point (8, 9, 32). This approach, however, often fails to prevent in vivo proteolytic degradation of the desired peptide, which is likely to be in an unfolded, protease-accessible conformation; nor does it allow for such degradation to be monitored before attempts to recover the peptide have been made. Furthermore, the carboxyl-terminal

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**Fig. 5. Cation exchange chromatography of V8 digest of AP-dynorphin.** A sample of AP-dynorphin V8 digest was applied to a 0.7 x 4-cm column of SP-Sephadex C-25 equilibrated with 50 mM phosphate buffer, pH 7.8. Bound material was eluted with a linear gradient of NaCl in the same buffer.

**Fig. 6. Analytical HPLC of the GPI-active SP-Sephadex fractions.** An aliquot (5%) of the pooled fractions displaying activity in the GPI assay from the SP-Sephadex C-25 chromatography shown in Fig. 5 was eluted on a C8 HPLC column with the linear acetonitrile gradient shown. A similar elution profile was obtained when the remaining material was eluted in the same way. The major component eluting at 27 min (24% v/v acetonitrile) was the desired dynorphin analogue, as determined by the analytical methods described in the text.
modification of proteins that are often used as carriers, such as β-galactosidase and even AP, often results in dramatic changes in their properties and intracellular compartmentalization, increasing the difficulty of extracting these hybrid proteins from cell lysates and recovering the desired peptide efficiently from them (32). The approach that we have described here offers several advantages. By inserting the dynorphin analogue that we wished to prepare into the AP structure, we have protected it from carboxypeptidase digestion and, at the same time, created a hybrid protein that may be readily monitored for degradation within the guima peptide segment at all times by electrophoresis in a denaturing gel. Also, by selecting a protein carrier for which a simple in vivo plate assay for enzyme activity is available, we have been able to screen many potential insertion points rapidly for their tolerance of such insertions. The retention of enzyme activity in the hybrid has two important advantages that are a consequence of the (presumed) correct folding and intracellular compartmentalization of the carrier protein: the conformation of the guest peptide will probably be constrained, as mentioned above, and therefore more resistant to proteases, and the properties of the hybrid protein are likely to be very similar to those of the unmodified carrier protein, allowing its recovery and purification by the same known methods, as in the present case. Finally, the selection of a protein that can be purified by anion-exchange chromatography as the carrier for a very basic peptide that is ultimately purified by cation-exchange chromatography allows simple, low cost, high yield techniques to be applied to the purification process without any need for affinity chromatography.

The successful identification of several surface-accessible sites in AP that tolerate 2- or 3-residue insertions, and our demonstration that both of the sites that were tested also tolerate the 15-residue dynorphin analogue insertion, suggests that surface insertion in AP may be a generally applicable approach to the useful preparation of any small peptide sequence through the in vivo expression of synthetic genes. Furthermore, the common occurrence of such insertion mutants in natural systems indicates that many other globular proteins might be effectively employed instead of AP as the carrier component. The major disadvantage of this approach relative to others results from the requirement for two sites of specific cleavage in order to excise the guest peptide for its recovery, but there is an ever increasing variety of solutions to this problem (32). We also expect that there may be a number of useful applications of this type of insertion mutagenesis involving, for example, the presentation on protein surfaces of antigenic determinants and other biologically active peptides, where excision of the guest peptide is not required, and we are currently exploring these possibilities.

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