Selective modification by transglutaminase of a glutamine side chain in the hinge region of the histidine-388→glutamine mutant of yeast phosphoglycerate kinase

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The transglutaminase-catalysed incorporation of putrescine and monodansylcadaverine into yeast phosphoglycerate kinase has been studied. There is little incorporation of the amines into wild-type enzyme, but nearly stoichiometric incorporation into the histidine-388→glutamine mutant enzyme. C.d. studies show that the overall structure of the mutant enzyme is very similar to that of the wild-type enzyme. Incorporation of the amines into the mutant enzyme causes no significant change in its activity. Glutamine-388 was shown, by isolation and sequencing of the modified peptide, to be the site of incorporation of monodansylcadaverine into the mutant enzyme. The specificity of the transglutaminase reaction is discussed in the light of available data.

INTRODUCTION

Transglutaminase (EC 2.3.2.13) catalyses the aminolysis of the γ -carboxamide group of the glutamine side chains of peptide and protein substrates. The reaction proceeds via an acyl-transfer mechanism in which the γ -carboxamide group acts as an acyl donor, and suitably unbranched primary amines act as acyl acceptors. *In vivo* the acyl acceptor is the ϵ -amino group of a lysine side chain, whereas in model reactions [14 C]putrescine or monodansylcadaverine are frequently employed as acceptors, since the reactions can then be readily monitored by the incorporation of radioactivity or fluorescence respectively. When putrescine is employed as acceptor, there is the possibility of cross-linking of protein substrates; this cannot occur in the case of monodansylcadaverine. Although a number of potential intracellular substrates (including cytoskeletal proteins such as tubulin and actin) have been identified from studies *in vitro*, the exact physiological role of the intracellular enzyme remains unclear (Zappia *et al.*, 1988). In the case of the serum enzyme (Factor XIIIa) an important substrate is fibrin and the cross-linking due to formation of the ϵ -(γ -glutaminyll)lysine isopeptide bond is presumed to be involved in the stabilization of the fibrin clot (Folk, 1983).

The transglutaminase-catalysed reaction offers a potential method for the selective introduction of functional groups into proteins under mild conditions. Although considerable work has been undertaken using model peptides to examine the sequence specificity around the glutamine side chain (Gorman & Folk, 1980, 1984), relatively little attention has been paid to the specificity in protein substrates. It had previously been suggested that conformational preferences may be the most important factor; i.e. that the glutamine should be in a flexible region of the polypeptide chain (Berbers *et al.*, 1983) or in a region with a propensity for reverse turns (Wold, 1985). As part of a programme in which we have been studying the transglutaminase-catalysed modification of enzymes of known structure, we

showed, in preliminary experiments, that none of the eight glutamine side chains in wild-type yeast phosphoglycerate kinase could be labelled to any significant extent. However, there is available a histidine-388→glutamine mutant (Wilson *et al.*, 1987) in which a glutamine side chain is placed in the hinge region connecting the two domains in the enzyme. (This mutant enzyme was prepared in order to explore the role of the Glu¹⁹⁰–His³⁸⁸ salt linkage in the catalytic mechanism.) The work reported here shows that Gln³⁸⁸ in the mutant enzyme can be modified in a specific manner. This opens up the possibility of introducing a 'reporter' (e.g. fluorescent) group into this region of the enzyme which might be useful in exploring the contribution of domain movements to the catalytic process (Wilson *et al.*, 1987).

MATERIALS AND METHODS

Materials

Enzymes and other reagents were purchased from the following sources. Transglutaminase (guinea-pig liver), trypsin [bovine pancreas, tosylphenylalanylchloromethane ('TPCK')-treated], monodansylcadaverine and putrescine dihydrochloride were from Sigma Chemical Co., Poole, Dorset, U.K. Guanidinium chloride (AristaR grade) and triethylamine (Hipersolv h.p.l.c. grade) were from BDH, Poole, Dorset, U.K. 1,4-[14 C]Putrescine dihydrochloride (4.07 GBq·mmol⁻¹) was from Amersham International plc, Amersham, Bucks, U.K. Cellulose filter discs (3 MM; 2.1 cm diameter) were from Whatman International Ltd., Maidstone, Kent, U.K. Propan-2-ol and acetonitrile (Far-u.v. h.p.l.c. grade) were from Labscan Ltd, Dublin, Ireland. Trifluoroacetic acid (h.p.l.c. grade) was from Rathburn Chemical Co., Walkerburn, Peebleshire, Scotland, U.K. Optiphase (M.P.) scintillation fluid was from FSA Laboratory Supplies, Loughborough, Leics., U.K. PD-10 (Sephadex G-25M) columns were from Pharmacia, Milton Keynes, Bucks., U.K. The Vydac C₁₈ h.p.l.c. column was from Technicol Ltd., Stockport, Cheshire,

U.K. Phosphoglycerate kinase (yeast) and glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle) were from BCL, Lewes, East Sussex, U.K. Other reagents were of the highest grade commercially available.

The wild type and His³⁸⁸→Gln mutant forms of phosphoglycerate kinase from yeast were prepared as described by Perkins *et al.* (1983) and Wilson *et al.* (1987). Plasmids pMA27 and pMA40b were kindly given by Dr. L. Gilmore, Department of Biochemistry, University of Edinburgh, Edinburgh EH8 9XD, Scotland, U.K. The kinetic properties of wild-type and mutant were identical with those reported by Wilson *et al.* (1987) when measured under the conditions used by them (i.e. in the mutant enzyme the k_{cat} and the K_m for ATP were lowered by factors of 5 and 3 respectively).

Enzyme assays

The concentrations of solutions of phosphoglycerate kinase (wild-type and mutant) were determined spectrometrically by using an $A_{280}^{1\text{ mg/ml}}$ value of 0.49 (Spragg *et al.*, 1976).

Phosphoglycerate kinase activity was assayed at 25 °C using the coupled assay involving glyceraldehyde-3-phosphate dehydrogenase. The final concentrations of reagents (in 0.1 M-triethanolamine/HCl adjusted to pH 7.6 with NaOH) were: ATP, 1.1 mM; 3-phosphoglycerate, 6.2 mM; NADH, 0.2 mM; EDTA, 0.9 mM; MgSO₄, 2 mM; and glyceraldehyde-3-phosphate dehydrogenase, 2.7 units/ml.

For storage and subsequent use the transglutaminase (2 units) supplied by Sigma as a freeze-dried powder was dissolved in water (1 ml) and stored in 0.1 ml portions at -20 °C.

Transglutaminase activity was monitored during the protein modification experiments using the colorimetric assay described by Folk & Cole (1965), in which the final concentrations of reagents (in 0.1 M-triethanolamine buffer, pH 7.6) are: benzyl-oxycarbonyl-Gln-Gly, 10 mM; hydroxylamine, 100 mM; CaCl₂, 5 mM; and dithiothreitol, 1 mM.

Modification of phosphoglycerate kinase

The transglutaminase-catalysed modification of phosphoglycerate kinase was carried out with the following concentrations of reagents in either 0.1 M-triethanolamine buffer, pH 7.6, or 20 mM-Tris/HCl, pH 7.6, at 25 °C: primary amine (putrescine or monodansylcadaverine), 5 mM; dithiothreitol, 1 mM; CaCl₂, 5 mM; phosphoglycerate kinase (2–4 mg/ml); transglutaminase, 0.1 mg/ml. The results obtained with the two buffers were, within experimental error, identical. In the case of putrescine, the [¹⁴C]putrescine was added to non-radioactive putrescine to give a final specific radioactivity of 9.25 MBq/mmol. Incorporation of putrescine was determined by liquid-scintillation counting after precipitation of protein on to cellulose discs by addition of 10% (w/v) trichloroacetic acid (Mans & Novelli, 1961). Incorporation of monodansylcadaverine was determined fluorimetrically (by reference to a standard curve) after separation of excess reagent by gel filtration through a Polydex column, using 1% (w/v) NH₄HCO₃ as the eluting solvent. Excitation and emission wavelengths were 320 nm and 520 nm respectively.

Sequence analysis of phosphoglycerate kinase

The cysteine side chain of native and of modified phosphoglycerate kinase carboxymethylated in 1% (w/v) NH₄HCO₃, pH 8.0, using the procedure of Allen (1981), i.e. incubation with 2 mM-dithiothreitol in 6 M-guanidinium chloride for 1 h, followed by an addition of a small molar excess (5 mM) of iodoacetate. After incubation for 1 h in the dark, the excess iodoacetate was allowed to react with excess 2-mercaptoethanol (200 mM). The carboxymethylated protein was then exhaustively dialysed against 1% (w/v) NH₄HCO₃, pH 8.0. Trypsin (1:40, w/w) was

then added and digestion was allowed to proceed for 6 h at 37 °C. After this time, a second portion of trypsin was added and the digestion continued for a further 6 h. Digestion was stopped by loading the sample on to a reverse-phase h.p.l.c. column as described below.

Separation of peptides in the trypsin digest was initially performed by reverse-phase h.p.l.c. on a Vydac C₁₈ (end-capped) column of dimensions 25 cm × 4.6 mm and particle size 5 μm. Peptides were eluted from the column by applying a linear gradient between 100% solvent A and 40% solvent A/60% solvent B over a period of 60 min. Solvent A consisted of aq. 0.4% triethylamine, adjusted to pH 2.5 with orthophosphoric acid. Solvent B was solvent A containing 60% (v/v) propan-2-ol.

In the case of phosphoglycerate kinase that had reacted with monodansylcadaverine, the fluorescence of the column eluate was monitored using excitation and emission wavelengths of 320 nm and 520 nm respectively. Fractions containing fluorescent material were further purified by h.p.l.c. on an Aquapore butyl B03Y02 column of dimensions 10 cm × 2.1 mm and particle size 7 μm. A linear gradient was applied over a period of 30 min between 100% solvent A [0.1% (v/v) trifluoroacetic acid in water] and 30% solvent A/70% solvent B. Solvent B consisted of 0.1% (v/v) trifluoroacetic acid in acetonitrile. The major peak (which contained over 80% of the applied fluorescent material) was then taken for protein sequencing and mass determination by fast-atom-bombardment m.s.

The sequence of the modified peptide was determined using an Applied Biosystems model-470 gas-phase sequencer with on-line 120A PTH analyser. Phenylthiohydantoin (PTH) derivatives produced at each cycle were identified by h.p.l.c. by reference to standard derivatives of amino acids.

Peptide mass determination was carried out on a VG Trio 3 Triple Quad mass spectrometer with an Ion Tech xenon fast-atom-bombardment gun. The scan mass range was from 1500 to 2500 Da, calibrated with caesium iodide clusters. Samples of 500 ng of modified peptide were dissolved in 5 μl of aq. 10% (v/v) acetic acid and 1 μl added to thioglycerol on the target.

C.d. spectra

C.d. spectra of phosphoglycerate kinase were recorded at 20 °C using a JASCO J-600 spectropolarimeter. The cell path-lengths for near-u.v. and far-u.v. spectra were 1 cm and 0.02 cm respectively, and protein concentrations were typically in the range 0.5–1.0 mg/ml. A detailed structural analysis of the enzyme required measurements in the far-u.v. down to 190 nm, and for this purpose the spectra were recorded in 25 mM-sodium phosphate buffer, pH 7.5. In a control experiment it was shown that the far-u.v. c.d. spectrum of the enzyme in one of the buffers used for the transglutaminase-catalysed reaction (i.e. 20 mM-Tris/HCl, pH 7.6) was identical with that observed in the phosphate buffer, at least down to 200 nm.

Molar ellipticity values were calculated using a value of 107 for the mean residue weight (Perkins *et al.*, 1983). The secondary-structure content was derived by using the CONTIN computer program (Provencher & Glöckner, 1981).

SDS/PAGE

Electrophoresis of protein samples was carried out on 12% (w/v) acrylamide gels by the method of Laemmli (1970).

RESULTS

Characterization of the wild type and mutant enzymes

The c.d. spectra of the wild-type and the His³⁸⁸→Gln mutant phosphoglycerate kinase are shown in Fig. 1.

Fig. 1. C.d. spectra of phosphoglycerate kinase

Spectra were recorded at 20 °C in 25 mM-sodium phosphate buffer, pH 7.5. (a) Far-u.v. spectra; (b) near-u.v. spectra. — and --- represent the wild-type and His³⁸⁸→Gln mutant enzymes respectively. The concentrations of enzymes were 0.5 mg/ml and 0.9 mg/ml in (a) and (b) respectively.

The far-u.v. c.d. spectra of the two enzymes (Fig. 1a) are virtually identical, confirming that they have very similar overall secondary structures. Using the CONTIN analysis (Provencher & Glöckner, 1981) of these spectra over the range 190–240 nm, the proportions of amino acids in α -helix and β -sheet structures can be evaluated. The results are $31 \pm 1\%$ α -helix for both enzymes and $33 \pm 1\%$ and $34 \pm 1\%$ β -sheet for native and mutant enzymes respectively. Comparison with the results from X-ray crystallography (Watson *et al.*, 1982) shows that the percentage α -helix is in good agreement (the X-ray results give 35% α -helix). However, the percentage β -sheet is considerably greater than that deduced from the X-ray data (13%). This discrepancy may partly arise from the contribution due to β -turns (12% in the X-ray structure), but it should be emphasized that the β -structure makes only a relatively small contribution to the c.d. spectra and thus estimation of β -structure is subject to greater error than that of α -helix.

The near-u.v. c.d. spectra (shown in Fig. 1b) confirm that the two enzymes possess very similar tertiary structures, although the dichroism of the mutant enzyme is slightly smaller than that of the wild-type. It is likely that the mutation in the hinge region and the consequent breakage of the salt-link between His³⁸⁸ and Glu¹⁹⁰ causes a small structural perturbation detected by neighbouring aromatic side chains. [Inspection of the X-ray structure of phosphoglycerate kinase (Watson *et al.*, 1982) shows that, for example, the side chains of Tyr¹⁹³ and Phe¹⁹⁴ are within a sphere of 0.75 nm radius from the α -carbon atom of His³⁸⁸ and that, in addition, the side chain of Trp³³³ is within a sphere of 1.0 nm radius.] Such a small perturbation would be consistent with the reduced specific activity of the mutant enzyme, which under our

conditions was 21 % that of the wild-type enzyme [a value similar to that previously reported (Wilson *et al.*, 1987)].

Transglutaminase-catalysed modification

The time course of incorporation of [¹⁴C]putrescine into wild-type enzyme is shown in Fig. 2(a). Only a small amount of incorporation (≤ 0.1 mol/mol) is observed over a 6 h period; this amount increased to about 0.2 mol/mol after 24 h. It is probable that this slow incorporation of [¹⁴C]putrescine may well reflect partial unfolding of phosphoglycerate kinase, since there

Fig. 2. Incorporation of [¹⁴C]putrescine into phosphoglycerate kinase

Reactions were carried out as described in the Materials and methods section. (a) Stoichiometry of incorporation. Δ and $\circ$ represent data for the wild-type and His³⁸⁸→Gln mutant enzymes respectively. In control experiments in which transglutaminase was omitted from the incubation mixtures, there was < 0.02 mol of putrescine/mol incorporated after 24 h. (b) Effect of incubation on the specific activity of phosphoglycerate kinase. Δ and $\circ$ represent data for the wild-type and His³⁸⁸→Gln mutant enzymes respectively. The corresponding closed symbols represent control mixtures from which transglutaminase was omitted. The initial specific activities under the conditions employed were 436 and 93 μ mol/min per mg for wild-type and mutant enzymes respectively.

Fig. 3. Purification by h.p.l.c. of peptides produced by tryptic digestion of the His³⁸⁸→Gln mutant of phosphoglycerate kinase

The modification reaction with dansylcadaverine catalysed by transglutaminase was performed as described in the Materials and methods section. (a and b) Separations of peptides on a Vydac C₁₈ column for modified (a) and unmodified mutant (b) enzymes. In each case approx. 0.2 mg of digested protein was applied to the h.p.l.c. column. In (a) the rectangles show the relative fluorescence of the fractions (excitation and emission wavelengths 320 nm and 520 nm respectively).

was a corresponding slow decline in the specific activity of the enzyme incubated under these conditions in the absence or presence of transglutaminase (Fig. 2b). Over this time period (24 h) there was no significant change in the near-u.v. c.d. spectra of wild-type enzyme incubated under these conditions. A control experiment using bovine pancreatic ribonuclease as substrate under these conditions showed that ≤ 0.05 mol of putrescine per mol of enzyme was incorporated after 24 h. This excludes the possibility that the low amount of incorporation noted above is due to autolabelling of the transglutaminase present.

When the mutant enzyme is used as the substrate for the transglutaminase-catalysed reaction, there is rapid incorporation of [¹⁴C]putrescine to an extent of 1.1 mol/mol after 6 h and to about 1.3 mol/mol after 24 h (Fig. 2a). There is a small decline in the specific activity of the mutant enzyme over this period (Fig. 2b), but this occurs both in the presence and in the absence of

transglutaminase, so is unlikely to be due to the modification reaction.

The incorporation of monodansylcadaverine into phosphoglycerate kinase was recorded after 20 h reaction, after removal of excess monodansylcadaverine by gel filtration. The extents of incorporation into wild-type and mutant enzyme were 0.3 and 1.26 mol/mol respectively, i.e. very similar to the stoichiometries of incorporation of putrescine. The effects on the catalytic activity in this case were very similar to those shown in Fig. 2(b) (when putrescine was used as the amine substrate).

SDS/PAGE of unmodified and modified (putrescine or monodansylcadaverine) enzymes showed that there was no detectable breakdown of the polypeptide chain during incubation for 20 h, i.e. that there was no degradation from any possible contaminating proteinases in the preparations.

Sequence analysis of the modified protein

Samples of unmodified and dansylcadaverine-modified phosphoglycerate kinase were subjected to digestion by trypsin as described in the Materials and methods section. The tryptic peptides were separated by reverse-phase h.p.l.c. (Fig. 3). Inspection of the peptide map for the modified mutant enzyme (Fig. 3a) shows that there is a distinct peak (labelled P1) containing the bulk of the fluorescence eluted at a position corresponding to 45% solvent B. This peak was not present in the tryptic digest of the unmodified mutant enzyme (Fig. 3b). It should be noted that the h.p.l.c. profile of the digest of the modified mutant enzyme displayed less-well-resolved peaks in the region corresponding to 35–40% solvent B than did the digest of the unmodified enzyme, suggesting that the presence of the large hydrophobic dansylcadaverine moiety may well inhibit the action of trypsin on neighbouring peptide bonds. However, the fact that a distinct new fluorescent peak was observed in the modified enzyme suggested that the major site of labelling was being examined.

Under these h.p.l.c. conditions, dansylcadaverine was eluted at a position corresponding to 20% solvent B; the small fluorescent peak observed at this position in the case of the modified mutant enzyme probably reflects a small amount of dansylcadaverine bound non-specifically to the protein even after prolonged dialysis.

The material corresponding to peak P1 was further purified by h.p.l.c. on an Aquapore butyl column, as described in the Materials and methods section. The main peak, corresponding to 32% solvent B, accounted for 90% of the applied material, as judged by A_{220} and showed the characteristic fluorescence of the dansyl moiety when spotted on polyamide plates and examined under u.v. light. The amino acid sequence of this main peak material was determined to be:

The repetitive yield for each cycle was $> 85\%$.

This sequence corresponds to the unique sequence of amino acids 386–403 in the published amino acid sequence of yeast phosphoglycerate kinase (Perkins *et al.*, 1983), with the unknown amino acid (Xaa) at the position corresponding to Gln³⁸⁸ in the mutant enzyme. The PTH derivative produced at the third cycle was freeze-dried and a portion was applied to a polyamide plate; this derivative showed the characteristic pale-green fluorescence of the dansyl group when excited by near-u.v. radiation.

As a final check that the dansylcadaverine moiety had been incorporated into the peptide in the mutant enzyme, the material in the main peak (from the Aquapore butyl column) was analysed by fast-atom-bombardment m.s. This showed that the parent ion had an M_r of 2062 as predicted from the sequence of amino acids

in the given peptide containing the monodansylcadaverine derivative of glutamine.

DISCUSSION

The results described here show that dansylcadaverine can be specifically incorporated into Gln³⁸⁸ of the His³⁸⁸→Gln mutant of yeast phosphoglycerate kinase, thus offering a means of introducing a reporter group into the hinge region of the enzyme. The increased hydrophobicity of the labelled peptide made it particularly convenient to achieve separation by h.p.l.c. The similarity of levels of incorporation of [¹⁴C]putrescine (Fig. 2a) makes it extremely likely that this is also incorporated specifically at Gln³⁸⁸ in the mutant enzyme, although this conclusion has not yet been confirmed by peptide isolation and sequencing.

Only very minor levels of dansylcadaverine could be incorporated into wild-type enzyme or into a number of other mutants, e.g. Arg¹⁶⁸→Met and Arg¹⁶⁸→Lys, which, as judged by c.d., are of similar overall structure (P. J. Coussons, C. M. Johnson, N. C. Price & S. M. Kelly, unpublished work). The lack of incorporation into wild-type enzyme is perhaps surprising in view of the fact that two of the eight glutamine side chains (Gln¹³⁵ and Gln¹⁴⁵) are in the exposed region ('nose') in the *N*-terminal domain (Watson *et al.*, 1982). However, it is possible that the large numbers of positively charged side chains around these two glutamine side chains could discourage the transglutaminase-catalysed reaction (cf. the sequence data in Table 1, discussed below). The c.d. spectra (Fig. 1) show that the His³⁸⁸→Gln mutant is of very similar overall secondary and tertiary structure to the wild-type enzyme, although there would appear to be a small local structural perturbation in the hinge region reflected in the near-u.v. c.d. spectrum (Fig. 1b) and the lowered catalytic activity. It is possible that, as a result of breaking the His³⁸⁸–Glu¹⁹⁰ salt linkage, a stretch of polypeptide chain including Gln³⁸⁸ in the mutant enzyme loops out so as to allow access to the active site of transglutaminase. As a result of modification of the mutant enzyme by incorporation of either putrescine or dansylcadaverine, there is no significant change in the catalytic activity (and, in the case of putrescine, in the c.d.

spectra). These observations would be consistent with Gln³⁸⁸ being in an exposed segment of polypeptide chain so that the amine moiety could be incorporated without significant structural consequences.

It remains to be established what in fact are the specificity requirements for transglutaminase-catalysed labelling of glutamine side chains in proteins. Table 1 shows the amino acid sequences around known sites of labelling in proteins. [In the cases of haemoglobin (α -chain) and insulin (A-chain), the proteins were denatured before labelling, whereas in the remainder they are believed to be in a native or near-native state during the labelling reaction, although in some cases this might be highly flexible.] A comparison of these sequences does not reveal particularly striking patterns, suggesting that the chemical nature of the side chains adjacent to the target glutamine may not be the sole determinant of specificity. A number of lines of evidence suggest that conformational factors may play an important role.

(i) In the case of actin, the major site of labelling has been identified as Gln⁴¹ (Takashi, 1988). From the 0.45 nm resolution X-ray data (Kabsch *et al.*, 1985) and from the results of limited proteolysis, Gln⁴¹ has been shown to occur in a surface loop.

(ii) Toda & Folk (1969) reported that almost all the glutamine side chains in a number of denatured proteins (including lysozyme, ribonuclease and ferredoxin) could act as substrates.

(iii) Using a multiple structure prediction approach (Sawyer *et al.*, 1988), we have examined the structural preferences of the sequences shown in Table 1. Apart from lipocortin I, for which α -helix structure is strongly predicted around the glutamine residue, it appears that the target glutamine side chains are generally in regions which are predicted to be reverse turns, at the end of β -sheet structure or to show no structural preferences.

It is, however, well recognized (Sawyer *et al.*, 1988; Schulz, 1988) that the prediction of reverse turns or loops is far less reliable than that of α -helical or β -sheet features. Thus it can be concluded that it will be necessary to include even more sophisticated tests of structure preference shown by amino acids (or sequences of amino acids) along with data on the active-site preferences of transglutaminase in order to improve the reliability of prediction or design of target sequences in proteins.

Table 1. Amino acid sequences around glutamine side chains known to be modified by transglutaminase

The site of labelling is indicated by the arrow.

Protein	Sequence	Reference
Lipocortin I	M V S E F L K Q A W F I E N E	Pepinsky <i>et al.</i> (1989)
β -Casein (succinylated)	D K I H P F A Q T Q S L V Y P E K A V P Y P Q R D M P I Q A I H P F A Q T Q S L V Y P F P Q N I P P L T Q T P V V V P P Q S V L S L S Q S K V L P V P S K V L P V P Q K A V P Y P Q I Q A F L L Y Q Q P V L G P V F M T S E K S Q T P L V T L F	Yan & Wold (1984)
β -Endorphin	D L S H G S A Q V K G H G K K	Porta <i>et al.</i> (1988)
Haemoglobin (heat-denatured, α -chain)	H S Q G T F T S D Y	Pincus & Waelsch (1968)
Glucagon	Y L D S R R A Q D F V Q W L M	Pincus & Waelsch (1968)
Insulin (isolated A-chain)	G I V E Q C C A S V C S	Mycek <i>et al.</i> (1959)
Actin	A S V C S L Y Q L E N Y C N	Folk & Cole (1965)
Collagen III aminopeptide	I V G R P R H Q G V M V G M G	Takashi (1988)
Phosphoglycerate kinase (His ³⁸⁸ →Gln mutant)	G G C S H L G Q S Y A D R D V G V T D K I S Q V S T G G G A	Bowness <i>et al.</i> (1987) The present work

We thank the Science and Engineering Research Council for provision of the c.d. facility and for the award of a CASE (Co-operative Awards in Science and Engineering) studentship to P.J.C., Dr. L. Gilmore and Dr. H. C. Watson for gifts of plasmids and over-expressing strains of yeast, Dr. S. W. Provencher for supplying the CONTIN computer program, Dr. A. F. W. Coulson for helpful discussions, and Mr. T. Gibson (Xenova Ltd.) for performing the fast-atom-bombardment m.s. and interpreting the results.

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Received 21 May 1990/30 July 1990; accepted 8 August 1990