MOLECULAR HETEROGENEITY OF ALKALINE PHOSPHATASE

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1. Introduction

Alkaline phosphatase (EC 3.1.3.1) from *E. coli* is a dimeric enzyme whose subunits are coded by the same gene [1]. Molecular heterogeneity of the subunits has been ascribed to differential modification of the N-terminus of each monomer after translation [2, 3]. In an attempt to verify this hypothesis we have looked for heterogeneity in the N-terminal sequence of the enzyme using an automatic sequenator [4].

2. Materials and methods

E. coli alkaline phosphatase (Worthington Biochemical Corp., New Jersey, USA, Grade BAPF) was dialysed against 0.1 M Tris-HCl, pH 8.0 to remove ammonium sulphate. Purity was assessed by electrophoresis on a 12% polyacrylamide gel containing 0.1% SDS [5], and on cellulose acetate strips. The enzyme was assayed by the method of Engstrom [6].

The N-terminus of the protein was determined by two methods of 'dansylation'. Firstly, according to Hartley [7], together with a non-hydrolyzed control to detect contaminating amino acids. Secondly, the dansylation was performed in 8 M urea. The dansyl protein was dialyzed against water before hydrolysis and the dansyl amino acids were identified on polyamide layers using the solvent systems previously described [7].

For sequence analysis 7.5 mg (200 nmoles subunit) of enzyme was carboxymethylated with 2-[^{14}C]iodoacetate [8] and then subjected to 20 cycles of Edman degradation using a Beckman 890B Sequencer. The standard double-cleavage programme was used. To improve adherence of the protein film a preliminary treatment with heptafluorobutyric acid, followed by chlorobutane wash, was included before beginning the degradation. The thiazolinones released were converted to PTH*-amino acids by heating at 80° under N_2 for 10 min in 1 M HCl (200 μl) containing 0.1% (v/v) ethanethiol. A 20 μl aliquot was removed for determination of S-[^{14}C]carboxymethylcysteine by liquid scintillation counting after each step in the degradation. After two extractions with 0.7 ml ethyl acetate, 2% (2–3 nmoles) of the extracted PTH-amino acid material was analysed on a Beckman GC-45 gas chromatograph. A further 2% was silylated 'on-column' [9] using *N,O-bis*(trimethylsilyl)acetamide as silylating agent. The remainder of each fraction (approx. 50–100 nmoles) was hydrolysed with HI (55% (v/v) aq. soln.) in a sealed-tube under vacuum at 130° for 20 hr. Regenerated amino acids were identified by amino acid analysis. Yields of PTH-amino acids were determined by measurements of GLC peak heights relative to a 5 nmole standard and by quantitation of the amino acid analyses.

3. Results

The sample of alkaline phosphatase showed a single protein band when examined by cellulose acetate and SDS-polyacrylamide gel electrophoresis. Enzyme assay showed a specific activity of 25 units/mg.

After 20 cycles of Edman degradation the N-terminal sequence of the protein was established as:

Arg–Thr–Pro–Glu–Met–Pro–Val–Leu–Glu–Asn–
Arg–Ala–Ala–Glu–Gly–Asp–Ile–Thr–Ala–Pro

* PTH = phenylthiohydantoin.

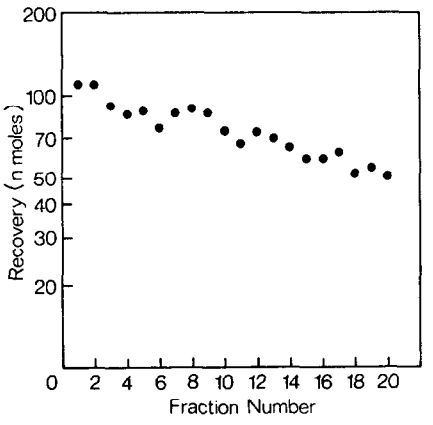

Fig. 1. Corrected semi-log plot of amino acid recoveries against fraction number. Yields of PTH-Ala, Gly, Val, Leu, Ile and Met were calculated from gas-chromatographic peak-heights. Arg, Pro, Glu and Asp yields were calculated from amino acid analyses. Threonine was estimated as α-aminobutyric acid after *HI* hydrolysis and amino acid analysis.

The yield of the principal amino acid at each step is shown in fig. 1. The repetitive yield was 97%. At step 20 overlap from the previous residue was 14% and the signal to noise ratio was 6:1. The sequence was unique and unambiguous from residue two, the minimum heterogeneity detectable being about 15%. The first fraction contained PTH-Gly, -Ala, -Ser, -Thr and -Arg but dansylation without hydrolysis indicated the first four of these to have arisen from contamination with free amino acids. Dansylation in 8 M urea followed by dialysis of the DNS-protein gave, after hydrolysis, DNS-arginine as the sole N-terminal residue. The yield of PTH-arginine at step one was 65% indicating that neither of the two chains was blocked.

4. Discussion

Piggot et al. [3] have found asparagine or aspartic acid, valine and threonine as N-terminal amino acids, in an alkaline phosphatase preparation shown to be heterogeneous by starch-gel electrophoresis. Natori and Garen [2] have isolated two N-terminal tryptic peptides from an ochre nonsense mutant of the same enzyme and found their partial sequences to be: His–Val–(?)–Ser–Arg and (?)–Ser–Arg. No other arginine-containing peptide was found. Both sets of authors suggest differential proteolysis at the N-terminus as a possible explanation of their heterogeneity. This could also explain the discrepancy between their observed N-terminal amino acids, the mutant protein comprising only 10% of the wild-type molecule probably having altered susceptibility to proteolytic attack.

In view of these results our demonstration that alkaline phosphatase from *E. coli* possesses a single N-terminal sequence over its first 20 residues is somewhat surprising. One possible explanation is that the commercial enzyme is isolated before any proteolysis has taken place. In this case at least 20 residues must have been removed from both forms of the ochre mutant since the peptide His–Val–(?)–Ser–Arg is not found in our sequence.

Alternatively, proteolysis proceeds to a defined point and our sequence represents the final homogeneous end-product. Since the remaining tryptic peptides isolated from the ochre mutant all contain lysine (except the C-terminal peptide which contained no basic amino acid) and our sequence contained no lysine the arginine of peptide His–Val–(?)–Ser–Arg can not correspond to our N-terminal arginine residue. Hence, at least 40 residues would have to be removed from the primary gene product and this would imply a considerably shorter wild-type molecule than that proposed by Natori and Garen. This hypothesis seems unlikely and we believe the first explanation to be correct, particularly as the mutant protein comprising only about 10% of the wild-type molecule, will differ in its susceptibility to proteolysis.

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