Journal of Chromatography, 326 (1985) 363–371
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 582

THE BACTERIAL PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEM

ISOLATION OF ACTIVE SITE PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND DETERMINATION OF THEIR PRIMARY STRUCTURE

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SUMMARY

Using reversed-phase high-performance liquid chromatography (HPLC) it was possible to isolate ^{32}P-labelled active-site regions of various proteins from the bacterial phosphoenolpyruvate-dependent phosphotransferase system. The purified peptides obtained by proteolytic cleavage with Lys-C protease and trypsin were sequenced by the gas phase method. The fragments derived from enzyme I (MW 70 000) of two streptococcal species show 100% homology. The analogous peptide of *Staphylococcus aureus* Enzyme I differs in the N-terminal region. A labelled peptide from the glucose-specific enzyme III protein of *Escherichia coli* obtained by cleavage with alkaline protease was isolated and sequenced. It could be fitted into the primary structure of this protein, which was derived from DNA sequence data. The active-site histidine residue of this protein is therefore localized at position 91. The HPLC separation method described is suitable for the isolation of peptides derived from active sites containing labile amino acid derivatives such as phosphohistidines.

INTRODUCTION

The bacterial phosphotransferase system (PTS) is a multienzyme system, involved in carbohydrate transport via vectorial phosphorylation[1]:

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$$\text{PEP} + \text{enzyme I} \xrightleftharpoons{Mg^{2+}} \text{P–enzyme I} + \text{pyruvate}$$
$$\text{P–enzyme I} + \text{HPr} \rightleftharpoons \text{P–HPr} + \text{enzyme I}$$
$$\text{P–HPr} + \text{III} \rightleftharpoons \text{P–III} + \text{HPr}$$
$$\text{P–III} + \text{sugar} \xrightarrow[\text{(enzyme II)}]{Mg^{2+}} \text{sugar–P} + \text{III}$$

During the above reaction sequence the phospho group of phosphoenolpyruvate (PEP) is first transferred to enzyme I (polypeptide of MW 70000, 3-P-histidine)[2,3]. In its next step, it is transferred to the histidine-containing phosphocarrier protein (HPr) (MW *ca.* 8000) and attached to His 15 at the N1-position of the histidine imidazole ring[4]. The phosphocarrier protein III is usually an inducible component, which specifically interacts with the membrane-bound inducible sugar-specific enzyme II. It is also converted into a phospho intermediate, usually phosphorylated at the N3-position of a histidine imidazole ring[3,5].

So far, attempts to demonstrate the type of phospho–histidine linkage have only been made by total hydrolysis of the protein with strong base or by NMR methods[6]. However, these methods do not allow the assignment of the active site within a primary structure. Isolation of the labile P–histidine-containing peptides has only been described for the lactose-specific III^{lac} protein of *Staphylococcus aureus* (MW of polypeptide chain, 12000)[6] where we succeeded in isolating a labelled active-site peptide by conventional chromatographic methods, starting from rather large amounts of material. In the case of the larger proteins, such as enzyme I, conventional methods were unsuccessful, because complex peptide mixtures resulted from proteolytic cleavage of this protein.

We therefore applied high-performance liquid chromatography (HPLC) to the isolation of ^{32}P-labelled peptides of the active site. An advantage of HPLC is the rapidity of separations, since the phospho–histidine bond is labile at neutral or acidic pH. We have determined the primary structure of the active-site peptides from enzyme I of *Streptococcus faecalis, Streptococcus lactis* and *Staphylococcus aureus* and of the glucose-specific III^{glc} componernt of *Escherichia coli,* which is involved in the transfer of the phospho group to glucose and has an additional regularoty function during inducer exclusion[7]. The aim of this study was to gain a deeper insight into the structures of this interesting transport system.

MATERIALS AND METHODS

Protein isolations

Enzyme I from *S. faecalis, S. lactis* and *S. aureus* was purified according to Alpert *et al.*[8]. The protein III^{glc} was isolated according to Dörschug *et al.*[9]. [^{32}P]PEP was synthesized as described previously[8].

Phosphorylation of PTS proteins with [^{32}P]PEP and tryptic cleavage

Phosphoenzyme I: isolation of active sites. A 1-mg amount of enzyme I (14 nmol), dissolved in 0.2 ml of 50 m*M* ammonium bicarbonate and 5 m*M* magnesium chloride (pH 8.5) was incubated with 0.1–0.5 μCi[^{32}P]PEP for 10 min at 37°C. Then

250 nmol cold PEP were added to achieve complete phosphorylation of enzyme I in 5 min at 37°C. The ^{32}P content of enzyme I was estimated by size-exclusion HPLC on a TSK W 125 column (300 × 7.5 mm) (Bio-Rad, Munich, F.R.G.).

The phosphoprotein was treated with one of the following proteases at a substrate to protease ratio of 20:1: trypsin, for 30 min; Lys-C and Arg C protease, for 90 min. Peptides were separated on a RP-8 column (10 μm) (Macherey Nagel, Düren, F.R.G.) by an acetonitrile gradient: solvent A, 20 m*M* potassium phosphate buffer (pH 6.8); solvent B, 85% acetonitrile (HPLC grade; Rathburn, U.K.). They were eluted with a linear gradient from 0 to 60% solvent B in 60 min at a flow-rate of 1 ml/min.

Radioactive fractions were collected manually and rechromatographed on the same column, equilibrated with 0.1% trifluoroacetic acid (TFA). They were eluted with a gradient of 0–60% solvent B in 60 min (solvent A, 0.1% TFA; B, 85% acetonitrile). The resulting fractions were collected and evaporated in a Savant Vacuum centrifuge (Uniequip, Munich, F.R.G.), yielding salt-free peptides suitable for amino acid analysis and automated Edman degradation in the liquid or gas phase[10].

Phospho-active site peptide. The phospho-III preparation required the phospho carrier protein HPr (*E. coli*) in addition to *E. coli* enzyme I. Phosphorylation of *E. coli* components with components from gram-positive bacteria is extremely slow. Protein III^{glc} (50 nmol), 2 nmol HPr, 100 nmol magnesium chloride and 50 pmol enzyme I in 40 μl of 50 m*M* ammonium bicarbonate (pH 8.3) were incubated with 10 μCi carrier-free [^{32}P]PEP in 50 μl of 0.2 *M* potassium phosphate buffer (pH 6.8). After 15 min at 37°C, 100 nmol PEP in 10 μl of 0.2 *M* Tris were added, and the mixture was incubated for another 5 min at 37°C. The [^{32}P]III^{glc} was isolated by passing the incubate through a Biosil TSK W 125 column (300 × 4.5 mm, Bio-Rad) with 0.1 *M* potassium phosphate buffer (pH 6.8) at a flow-rate of 0.7 ml/min. The radioactivity and UV absorption were recorded, the radioactive peak of MW 20000 was collected (1.5 ml) and 50 μg of subtilisin in 10 μl of 0.05 *M* ammonium bicarbonate (pH 8.3) were added and incubated for 1.5 h.

For alkaline protease digestion, *Streptomyces griseus* protease was used. The [^{32}P]III^{glc} solution was adjusted to pH 11 with 1 *M* potassium hydroxide and 50 μg of protease in 10 μl of 0.05 *M* ammonium carbonate, pH 8.3 were added, followed by incubation at 37°C for 2 h. The whole mixture was applied to a Nucleosil C_8 column (250 × 4.6 mm), and the peptides were isolated by gradient elution, as described for the separation of enzyme I peptides. Again, two reversed-phase analyses were performed at pH 6.8 and 2.1, respectively. Peptides were detected by their UV absorption at 230 nm, and their radioactivity was monitored by flow-through counting.

Proteases

Worthington trypsin N-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-treated was purchased from Cooper Biomedical (Frankfurt, F.R.G.). Lys-C protease, Arg-C protease and subtilisin were purchased from Boehringer (Mannheim, F.R.G.). *Streptomyces griseus* protease was purchased from Calbiochem (Frankfurt, F.R.G.).

HPLC equipment

The HPLC equipment consisted of the following components: a M 250 B low-

pressure gradient former (Gynkothek, Munich, F.R.G.); a Jasco Twincle Pump (Biotronik, Munich, F.R.G.); a Rheodyne 7125 syringe sample injector (Biotronik); a column oven (Knauer, Berlin, F.R.G.); a Jasco Uvidec III UV monitor (Biotronik) and a Berthold LB 504 radioactivity flow-through monitor (Berthold, Wildbad, F.R.G.), attached to an Apple IIe computer.

The ^{32}P activity was measured by Čerenkov radiation in a loop of PTFE tubing (volume 0.2 ml).

Reversed-phase columns (250 × 4.6 mm were packed with Nucleosil C_8, 10 μm (Macherey Nagel) by Bischoff Analysentechnik (Leonberg, F.R.G.).

RESULTS AND DISCUSSION

Active-site peptides of enzyme I from various Gram-positive microorganisms

Phosphorylation of enzyme I with ^{32}P-labelled PEP could readily be performed in the presence of Mg^{2+}. Proteolytic cleavage with the proteases used astonishingly occurred without prior denaturation at physiological pH values, yielding almost quantitatively a single radioactive peptide for all enzyme I proteins studied (Fig. 1) after separations performed at pH 6.8. Further purification of the labelled enzyme I active-site peptides at pH 2.1 in 0.1% TFA did not result in a major loss of the ^{32}P label by hydrolysis. This readily occurs with the phospho–histidine linkage present in the native PTS phosphoproteins[5] (Figs. 2, 3).

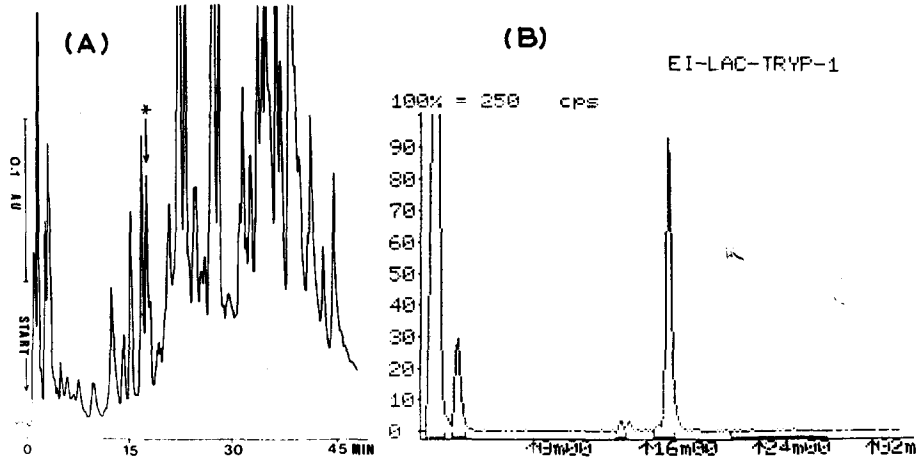

Fig. 1. A, Gradient elution of tryptic peptides of ^{32}P-labelled phospho–enzyme I from *Streptococcus lactis* on an RP 8 column at pH 6.8. The asterisk indicates the position of labelled peptide in the UV-track. B, Distribution of ^{32}P in chromatogram A.

One plausible explanation for this unexpected behaviour may be that the phosphopeptides were denatured under the chromatographic conditions. We observed a similar reduction of the rate of hydrolysis of ^{32}P-labelled PTS proteins after treatment with the denaturing agent sodium dodecyl sulphate. Another factor in the stability of the phospho–histidine linkage in the enzyme I active-site peptides is the type of bond, which very likely occurs at the 3 position of histidine, as demonstrated for the *S. aureus* enzyme I[3].

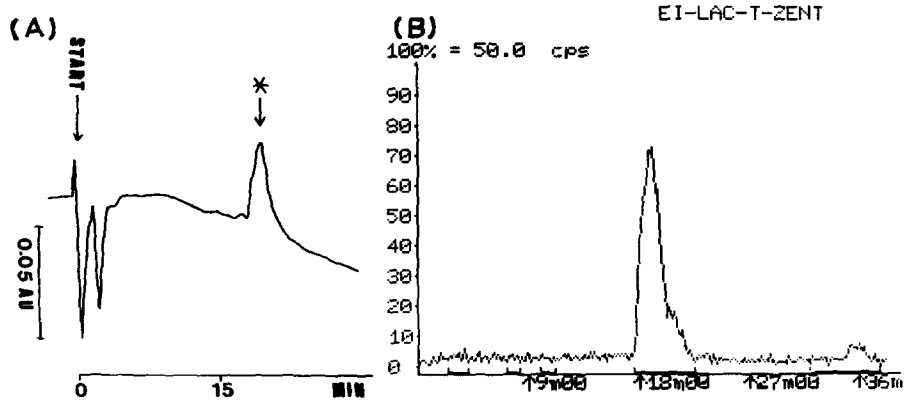

Fig. 2. A, Rechromatography of the radioactive peak at pH 2 (0.1% TFA) on the same RP-8 column as in Fig. 1: UV absorption at 230 nm. The asterisk indicates the position of ^{32}P label. B, Radioactivity distribution in chromatogram A.

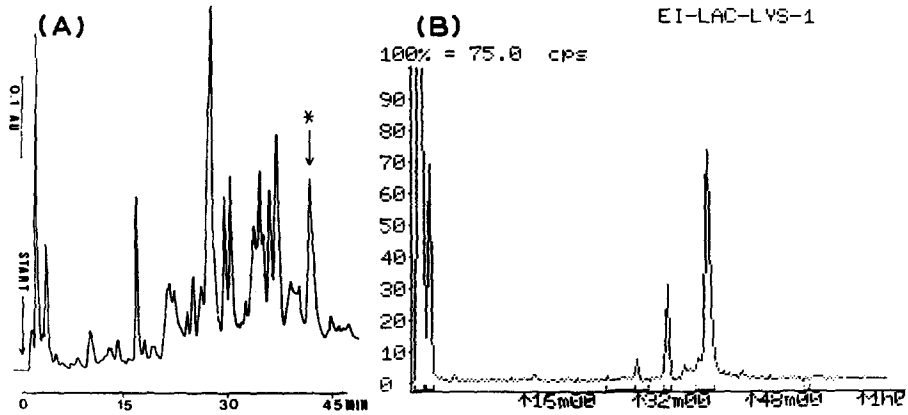

Fig. 3. A, Gradient elution of Lys-C proteolytic fragments of ^{32}P-labelled phospho-enzyme I (*Streptococcus lactis*) on an RP-8 column. The asterisk indicates the position of ^{32}P label in the UV-track. B, Distribution of the ^{32}P label.

Fig. 1 is an impressive example of the separating power of reversed-phase HPLC for peptide mixtures. According to amino acid analysis, the enzyme polypeptide chain contains 63 lysine and arginine residues, resulting in a minimum of 64 tryptic peptides (Table I). Only two chromatographic steps were required to isolate pure peptides in yields sufficient to obtain the amino acid sequence from 1 mg of starting material.

The active-site peptide from the III^{glc} component of E. coli

Proteolytic cleavage with the site-specific proteases trypsin, Arg-C protease and Lys-C protease, which readily digested enzyme I proteins, was not very effective for III^{glc} protein. Cleavage occurred only after prolonged incubation times, but was accompanied by loss of the ^{32}P label from the peptide. One explanation for this may be the high stability of the tertiary structure of this protein, already observed during

TABLE I

AMINO ACID COMPOSITION OF ENZYME I FROM *STREPTOCOCCUS FAECALIS* AND FROM *STREPTOCOCCUS SALIVARIUS*[17]

Tryptophan was determined according to Spande *et al.*[18].

Amino acid	*S. Salivarius*	*S. faecalis**
Aspartic acid	64	75
Threonine	39	37
Serine	31	29
Glutamic acid	82	85
Proline	15	25
Glycine	46	43
Alanine	79	77
Valine	44	53
Cysteine	6	Not determined
Methionine	16	22
Isoleucine	32	31
Leucine	55	52
Tyrosine	13	20
Phenylalanine	21	19
Histidine	11	6
Arginine	27	27
Tryptophan	Not detected	2
Lysine	37	36

* Amino acid composition determined according to ref. 16.

NMR measurements[11]. The relatively non-specific protease, subtilisin, produced a hexapeptide in an amount too small to determine the amino acid sequence. Good results were obtained with the alkaline protease from *Streptomyces griseus*, which has the advantage of attacking the peptide linkage at pH 11, where phosphohistidines are expected to be very stable. Using this protease, a peptide with the sequence Val-His-Phe-Gly-Ile-Asp was isolated under similar separating conditions to those used for the enzyme I active sites (Figs. 4 and 5). Since the amino acid sequence derived from the DNA sequence had been worked out for III^{glc} of *E. coli*[12] and *Salmonella typhimurium*[13], it was possible to recognize the active site of III^{glc} as histidine 91. In *S. aureus* III^{lac}, His 82 has been demonstrated to be the phosphorylation site[14].

Similarities of the active sites of S. faecalis, S. lactis and S. aureus enzyme I and of various III proteins

The sequences determined (Table II) show almost 100% homology for *S. faecalis* and *S. lactis* enzyme I active-site peptides. According to its amino acid composition enzyme I of *Streptococcus salivarius*[17] is closely similar to the enzyme I of *Streptococcus faecalis* (Table I). We therefore would predict that the active-site regions of this enzyme I should also show a high degree of homology. This was not unexpected, because very good homology between the HPr proteins of the PTS of these two organisms were detected by comparison of the NMR spectra[4]. However, the *S. aureus* enzyme I is definitely different: even the preliminary data now available show that the first residues of the Lys-C peptides are not homologous.

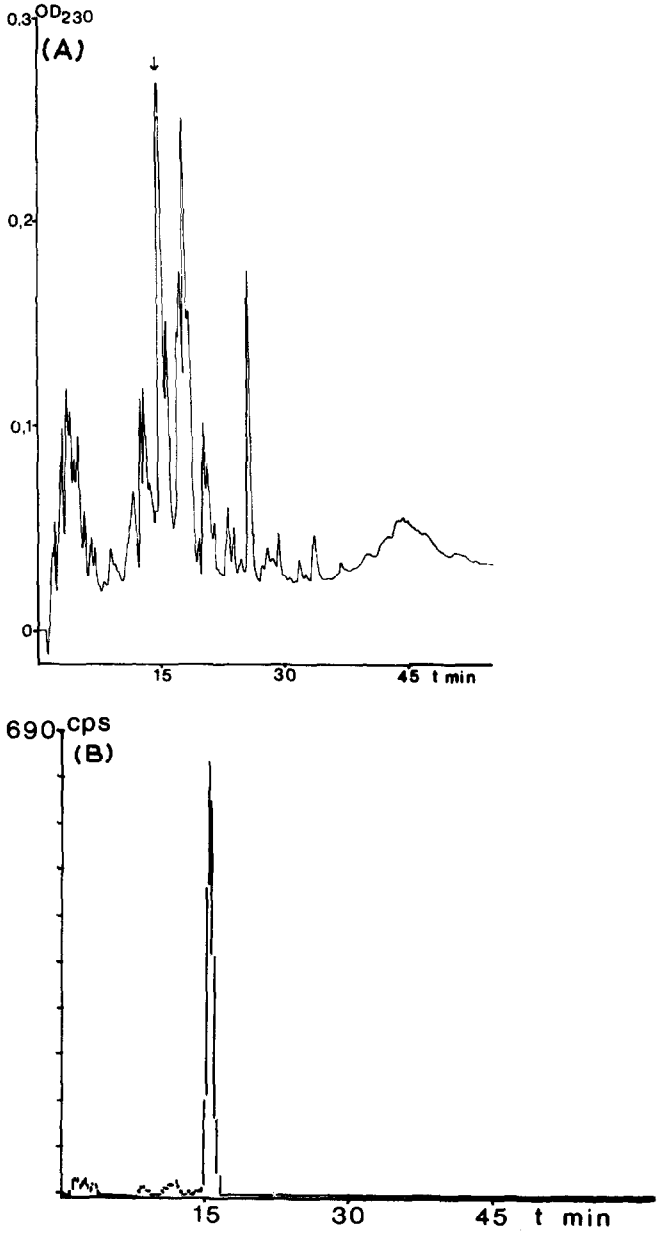

Fig. 4. A, Gradient elution on a RP-8 column at pH 6.8 of peptides, obtained by cleavage of ^{32}P labelled phospho-III^{glc} (*Escherichia coli*) with alkaline protease from *Streptococcus griseus*. The arrow indicates the position of labelled peptide. B, Distribution of ^{32}P on chromatogram A.

To summarize, *S. aureus* PTS proteins are different from the corresponding streptococcal proteins, but the similarities in tertiary structures must be extensively conserved, since staphylococcal and streptococcal proteins are interchangeable in the biological assay[15]. As mentioned earlier, *E. coli* PTS proteins operate only very in-

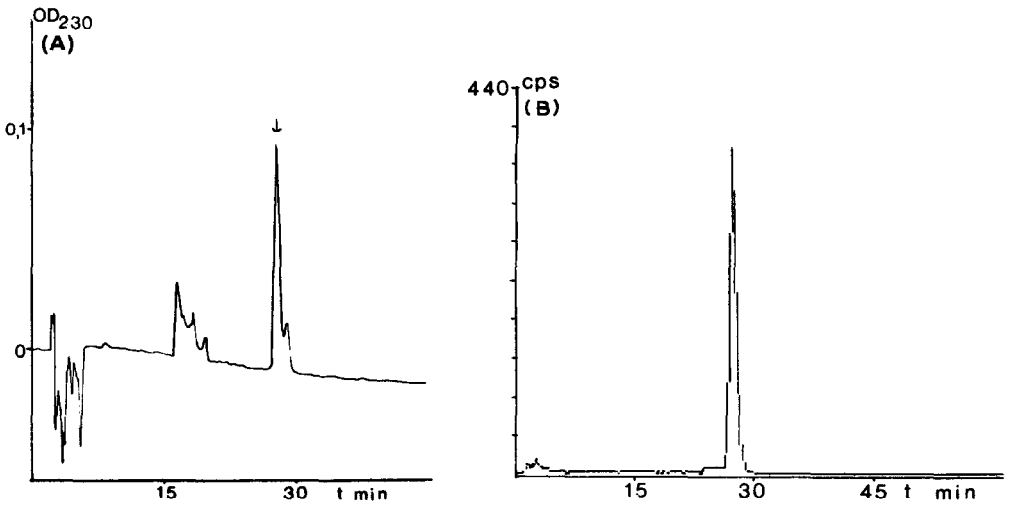

Fig. 5. A, Rechromatography of the labelled peptide at pH 2.0 on the RP-8 column. The arrow indicates the position of labelled peptide. B, Distribution of ^{32}P corresponding to chromatogram A.

TABLE II

PRIMARY STRUCTURE OF ACTIVE-SITE PEPTIDES OF ENZYME I FROM VARIOUS GRAM-POSITIVE MICROORGANISMS

Organism	*Structure*
S. faecalis	-Ala-Phe-Val-Thr-Asp-Ile-Gly-Gly-Arg-Thr-
S. lactis	-Ala-Phe-Val-Thr-AsN-Ile-Gly-Gly-Arg-Thr-
S. aureus	-Glu-Phe-Val-GlN-Gly-Phe-Ala-
S. faecalis	-Ser-His-Ser-Ala-Ile-Met-Ala-Arg-Ser-Leu-
S. lactis	-Ser-His-Ser-Ala-Ile-Met-Ala-Arg-Ser-Leu-
S. aureus	
S. faecalis	-Glu-Ile-Pro-Ala-Ile-Val-Gly-Thr-Lys-
S. lactis	-?-Ile
S. aureus	

efficiently in the staphylococcal PTS assay system. Whereas the active-site regions of gram-positive and gram-negative HPr proteins are rather similar, no homology can be detected between III^{lac} of *S. aureus* and III^{glc} of *E. coli*. Further studies of the active sites of PTS proteins should be performed to detect the general features of the primary structure of these proteins.

In the case of the HPr proteins, the active site has been proposed to be a protrusion of the protein[16]. For the enzyme I and factor III proteins one would expect the active site to be a cleft which recognizes the protrusion of HPr. It is plausible to assume that the active site, located on a peptide loop, must be formed by a relatively short segment of the polypeptide chain, whereas a cleft is usually constructed from peptide segments which may be separated by many residues. There-

fore, catalytically important residues may well be far apart, in terms of the primary structure, but close together in terms of the tertiary structure.

CONCLUSION

In recent years a large number of primary structures of proteins have been worked out by DNA sequencing; the extent of this type of information is still growing. However, this structural information becomes valuable only when the regions of interest, such as the active sites, can be assigned within the polypeptide chain. We believe we have demonstrated that HPLC separation of peptides is a very efficient biochemical tool for supplying information about active sites. DNA sequencing, in conjunction with the isolation of active-site regions, NMR spectroscopy and X-ray crystallography, should lead to a rapid increase in precise structural information on PTS components and on proteins in general, even if rather labile intermediates are involved in the catalytic mechanism.

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