A Biologist's Guide to Principles and Techniques of Practical Biochemistry

Third Edition

Edited by
Keith Wilson

B.Sc., Ph.D.
Head of Division of Biological and Environmental Sciences,
The Hatfield Polytechnic

and
Kenneth H. Goulding

M.Sc., Ph.D.
Head of School of Applied Biology,
Lancashire Polytechnic

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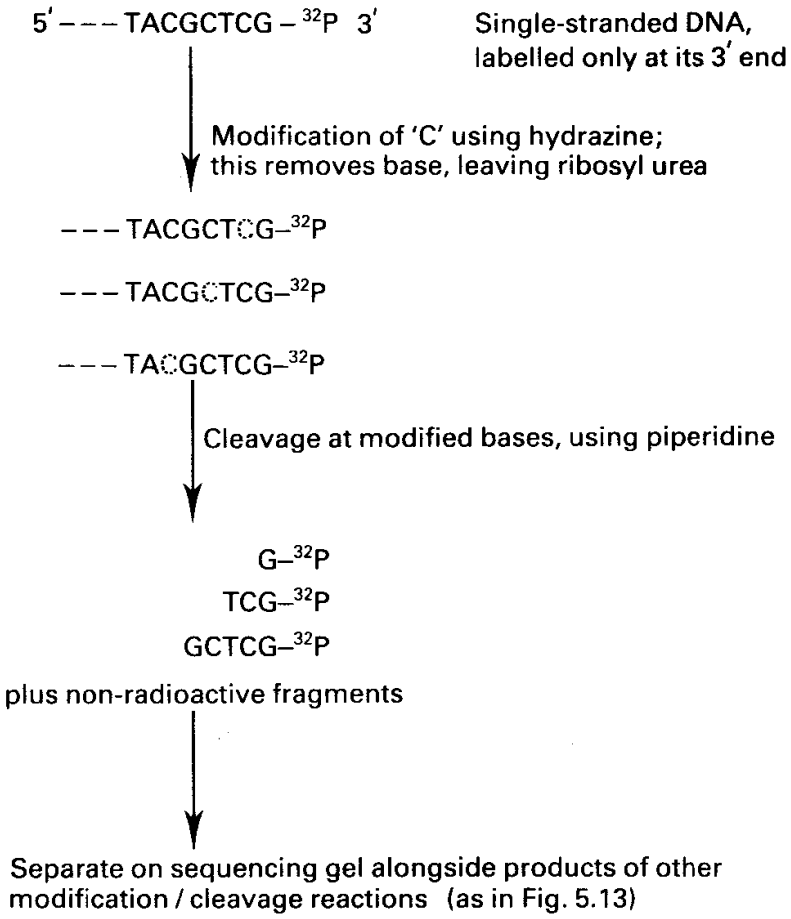

Fig. 5.14 Maxam and Gilbert sequencing of DNA. Only modification and cleavage of deoxycytidine is shown, but three more aliquots of the end-labelled DNA would be modified and cleaved at G, G + A, and T + C, and the products would be separated on the sequencing gel alongside those from the 'C' reactions.

to that produced by the Sanger method, since each sample now contains radioactive molecules of various lengths, all with one end in common (the labelled end), and with the other end cut at the same type of base. Analysis of the reaction products by electrophoresis is as described for the Sanger method.

Because the Sanger method produces oligonucleotides which are radioactively labelled throughout their lengths, rather than only at one end, the molecules can be made a lot more radioactive, and therefore easier to detect; so less DNA is needed for sequencing. Once M13 cloning has been set up in a laboratory, it provides a very convenient and rapid way to obtain single-stranded DNA. For these reasons, dideoxy sequencing of M13-cloned DNA is probably the most commonly used sequencing method, though the chemical procedure is still used by many laboratories.

5.5.3 Protein sequencing

Although protein sequencing may seem out of place in a section dealing with the analysis of DNA, the molecular biologist can often make use of a

knowledge of protein sequences when manipulating DNA. If the sequence of a protein is known, a gene coding for it can be synthesised chemically (though this is usually only worth doing for small polypeptides), or an *oligonucleotide probe* can be synthesised for use in recovering the gene for that protein from a *gene library* (Section 5.9.5).

Since it is currently impossible to sequence a polypeptide longer than about 100 amino acids, pure proteins must be fragmented to give polypeptides of a length which can be sequenced, and these polypeptides must be separated from each other prior to sequencing. Fairly specific and limited cleavage can be obtained by chemical means. For example, cyanogen bromide cleaves only at (rare) methionine residues, BNPS-skatole cleaves at tryptophan, and hydroxylamine breaks the linkage between asparagine and glycine. Similarly, several proteolytic enzymes, such as trypsin and V8-Protease, have a fairly specific site of action, and will therefore generate relatively few cleavage products.

The polypeptides so produced are separated from each other prior to sequencing, using such techniques as exclusion chromatography (Section 6.6) or HPLC (Section 6.8). Relative positions of the polypeptides within a protein can be found by looking for overlaps in the sequences of polypeptides generated by different means, and in this way the entire protein sequence may be deduced.

All protein sequencing methods are based on the *Edman degradation* of polypeptides, in which the *N*-terminal amino acid is specifically removed, leaving a polypeptide one amino acid residue shorter. Variations arise in the method of identifying the removed amino acid or the newly exposed *N*-terminal amino acid. By repeated cycles of Edman degradation and identification of product, the polypeptide can be sequenced.

In the *Edman reaction* (Fig. 5.15) the polypeptide is treated with phenylisothiocyanate (PITC), which reacts with the *N*-terminal amino acid to form a phenylthiocarbamyl (PTC) derivative of the polypeptide. Anhydrous trifluoroacetic acid is then used to cleave the molecule, giving the 2-anilino-5-thiazolinone derivative of the *N*-terminal amino acid and also the polypeptide shortened by one residue. The thiazolinone derivative is separated from the polypeptide and converted into the more stable 3-phenyl-2-thiohydantoin (PTH) derivative, which is then identified by HPLC or TLC. By repeating this cycle the polypeptide can be sequenced from its *N*-terminal end. The process has been automated, either by immobilising the protein on an inert, solid support (*solid-phase sequencers*), or by keeping the protein spread out in a thin film for maximum exposure to reagents (*spinning cup sequencers*). Such instruments can, under ideal conditions, sequence up to 100 residues of a protein.

The alternative *Dansyl-Edman* procedure (Fig. 5.16) is highly sensitive, allowing as little as 1 nmole of polypeptide to be sequenced, and it is therefore well suited to manual determination of sequences. It uses cycles of the Edman reaction to remove *N*-terminal amino acids sequentially, but, instead of identifying the released PTH derivatives, it identifies the newly exposed *N*-terminal amino acids. This is achieved by adding a dansyl group to the *N*-terminal of a very small sample of the polypeptide after each cycle of the

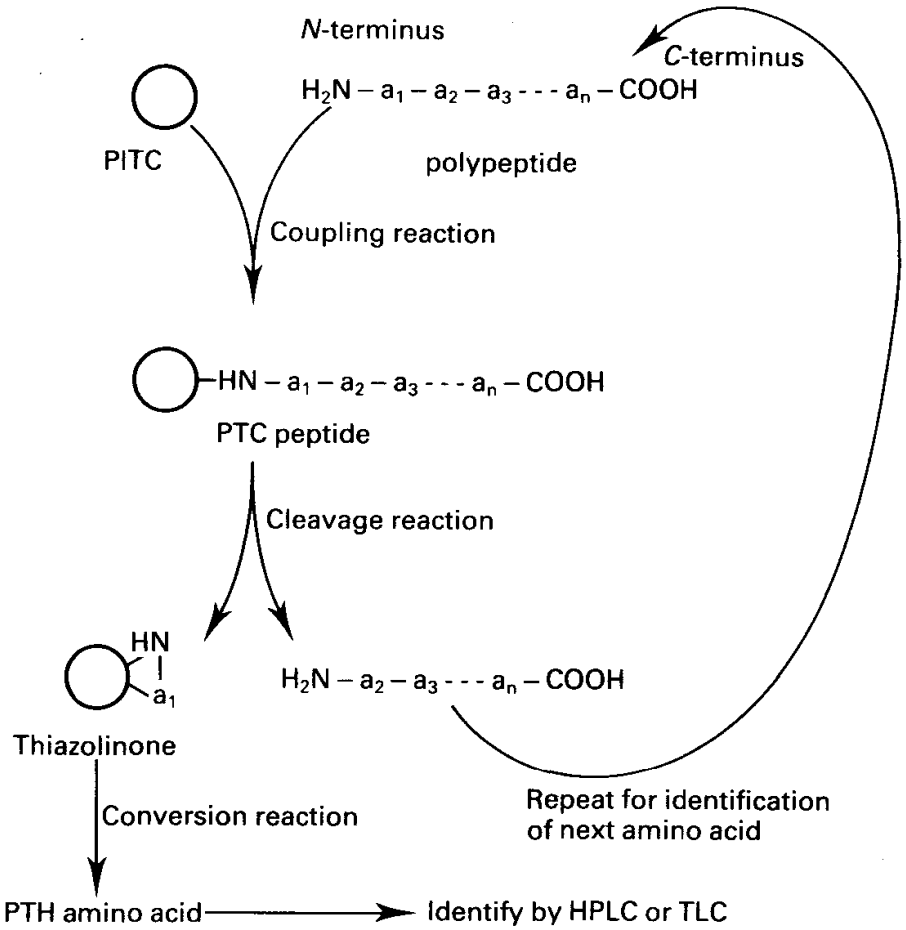

Fig. 5.15 Edman reactions. PITC phenylisothiocyanate; PTC, phenylthiocarbamyl; PTH 3-phenyl-2-thiohydantoin. Note that each cycle of reactions removes one amino acid from the *N*-terminus of the polypeptide.

Edman reaction, followed by cleavage with hydrochloric acid to release a dansyl amino acid plus free amino acids. The dansyl derivative can be identified by two-dimensional TLC on polyamide plates (Section 6.1.3). Up to about 15 amino acids can be sequenced before the cumulative effects of incomplete reactions and side reactions make impossible the unambiguous identification of the dansyl amino acid.

Given the nucleotide sequence of a gene, and our knowledge of the genetic code, it is easy to read off the amino acid sequence for which the gene codes, provided the correct reading frame is used, and the sequence is not interrupted by introns. Ironically, DNA sequencing, rather than protein sequencing, has sometimes been used to obtain amino acid sequences of proteins, especially when the pure protein has not been obtainable in sufficient quantities for direct sequencing. However, it should be remembered that a lot of effort is involved in the isolation of a specific gene, and this may more than offset the rapidity of DNA sequencing. The pace of sequencing is such that computers are now used by some laboratories for the analysis of sequencing gels, and sequence data banks have been set up to cope with the massive flow of information. In spite of this, it will be some time before the human genome

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