A SIMPLIFIED ROUTE TO [^{14}C] DIMETHYLSUBERIMIDATE FOR POLYPEPTIDE CROSSLINKING STUDIES

Synthesis of [^{14}C]suberonitrile by phase transfer catalysis using 18-crown-6 ether

L. C. PACKMAN and D. J. H. SMITH+

Department of Biochemistry and +*Department of Chemistry, University of Leicester, Leicester, LE1 7RH, England*

Received 15 May 1978

1. Introduction

Crosslinking reagents have become valuable tools in the study of the interactions between subunits in multimeric proteins [1], in determining the proximity of segments of the same polypeptide chain [2] or, as in the study of prokaryotic ribosomes, the interactions between contiguous proteins [3]. The analytical potential of these reagents in assessing the symmetry of tetrameric enzymes, for which X-ray diffraction data may not be available, has already been demonstrated [4]. Although the success of the method is clearly limited by the ease with which crosslinking can occur in any given system, the method has general applicability.

In the study of proteins of known primary structure, the use of crosslinking reagents may be extended to the isolation and identification of the amino acid residues involved in crosslink formation and their location in the polypeptide chain [5,6]. The use of a radioactive reagent is clearly useful and often essential as a convenient means for monitoring crosslinked peptides during purification of these species from enzymic digests of crosslinked proteins.

In the synthesis of bis-imido-esters, the corresponding dinitrile precursors are generally unavailable in radioactive form, and their generation from the dihaloalkane and potassium or sodium [^{14}C]cyanide has involved the use of high temperatures in the displacement reactions [7,8] and distillation of the product from the various solvents [8,9].

This paper describes a high-yield synthesis of [^{14}C]-suberonitrile using an 18-crown-6 phase transfer catalysed reaction and the use of this product to synthesise [^{14}C]dimethylsuberimidate dihydrochloride. Using this method, the potential hazards of previously reported syntheses are minimised, and the overall procedure is within the scope of many laboratories which are unaccustomed to radioactive organic syntheses.

2. Materials and methods

1,6-Dichlorohexane and 18-crown-6 ether were products of Aldrich Chemical Co. England; K^{14}CN was obtained from the Radiochemical Centre, Amersham; Sequencer grade acetonitrile was from Pierce Chemical Co., IL. Sequenal grade methanol was from Rathburn Chemicals, Scotland; hydrogen chloride (99%) was from Cambrian Chemicals Ltd., Croydon; and bovine liver catalase (2 X crystallised) and triethanolamine hydrochloride were obtained from Sigma Chemical Co. Ltd., Surrey. All other chemicals used were analytical grade. Dimethylsuberimidate dihydrochloride, prepared from suberonitrile by the method in [1] was a generous gift of Dr J. R. Coggins, University of Glasgow.

2.1. *Synthesis of [^{14}C]suberonitrile*

K^{14}CN, 3 mCi, were mixed with finely ground KCN 1.37 g (21 mmol) and dried overnight at 105°C. After cooling over desiccant, the powder was suspended in 7 ml anhydrous acetonitrile containing 525 mg

Elsevier/North-Holland Biomedical Press

(2 mmol, 10 mol%) of 18-crown-6 ether. Dichlorohexane 1.55 g (10 mmol) were added with stirring and the vial was flushed with dry nitrogen and sealed. The reaction mixture was stirred vigorously at room temperature for 40 h, and then poured into 100 ml water. This was extracted 3 times with 50 ml dichloromethane and the organic fractions were combined, dried over anhydrous sodium sulphate and finally concentrated to constant weight under reduced pressure (30 mm) at 50°C to remove solvent. The temperature was then raised briefly to 80°C to ensure complete removal of acetonitrile.

The resulting [^{14}C]suberonitrile (96% yield) containing residual dichlorohexane was used for the synthesis of [^{14}C]dimethylsuberimidate by a slight modification of the method in [1].

2.2. *Synthesis of [^{14}C]dimethylsuberimidate dihydrochloride*

[^{14}C]Suberonitrile (9.6 mmol, 1.31 g) was cooled to 0°C and an ice-cold solution of anhydrous methanol (1.6 ml, 40 mmol) in anhydrous ether (5 ml) was added under anhydrous conditions. This mixture was saturated with anhydrous hydrogen chloride and incubated at 0°C for 24 h. [^{14}C]Dimethylsuberimidate was crystallised out by addition, in 10 ml aliquots, of 50 ml anhydrous, ice-cold ether with mixing. The mixture was then incubated in a solid-CO_2/acetone bath for 30 min to maximise the crystallisation yield. On warming to 0°C, the crystals were collected by careful decantation and washed extensively with anhydrous ether and finally dried under vacuum, yielding 2.5 g (9.2 mmol, 92% overall yield) product.

[^{14}C]Dimethylsuberimidate was seen to be identical with a pure non-radioactive sample on examination by NMR spectroscopy and had spec. act. 0.25 mCi.mmol^{-1}. Radiochemical yields were generally lower than chemical yields presumably due to the variable physical nature of the K^{14}CN. A request for finely ground material is recommended to ensure high radiochemical yields.

2.3. *Crosslinking of catalase*

The functional properties of the product were tested in parallel with non-radioactive dimethylsuberimidate and also a commercially obtained sample (Pierce). Stock solutions (1 M) of the crosslinkers in 0.2 M triethanolamine hydrochloride, pH 8.5, containing 1 M NaOH were prepared immediately before use. Crosslinking of catalase (0.5 mg.ml^{-1}) [4], which was exhaustively dialysed against 0.2 M triethanolamine hydrochloride, pH 8.5, was carried out at room temperature for 3 h using final conc. 20 mM (5.5 mg.ml^{-1}) dimethylsuberimidate.

Amidination was quenched by addition of equal vol. 0.1 M–Tris.HCl, pH 6.8 and, after 30 min, an aliquot (40 μg) was incubated at 100°C for 2 min in the presence of 1% (w/v) SDS and 2-mercaptoethanol (0.2 M) prior to electrophoresis in an SDS–polyacrylamide slab gel (8% acrylamide monomer, 2.6% crosslinking) using the buffer system of Laemmli [10].

Gels were stained overnight with a solution of 0.00125% (w/v) Coomassie brilliant blue R (Sigma) in methanol/acetic acid/water (40:7:53, by vol.) and destained in 7% (v/v) acetic acid.

Radioactivity in the protein bands was detected by fluorography at –70°C for 7 days [11].

3. Results and discussion

In synthesising radioactive suberonitrile on a scale involving mCi amounts of label, it is desirable to employ a method which may reduce potential operational hazards to a minimum, while maintaining high yields of product in a pure form. Previously reported methods for the synthesis of [^{14}C]aliphatic dinitriles [5–7] have involved the use of dimethylsulphoxide as solvent and reaction temperature of 90–130°C. The alternative use of polyethyleneglycol 300 [8] produces a reaction of similar efficiency (75–90%) but the dangers of violent reaction with increasing chain length are clearly undesirable. While reaction times are generally short (15–30 min), separation of the dinitrile from the solvent has required distillation under reduced pressure (10–15 mm) at 150–200°C, a step which could prove difficult for malononitrile synthesis where the boiling points of the solvent and product are separated by less than 30°C.

The method described here, which is equally applicable to the synthesis of diimidoesters of different chain lengths, overcomes most of these difficulties by use of acetonitrile as an aprotic solvent and 18-crown-6 macropolycyclic ether [12] as a phase transfer catalyst. (Readers are directed to [13] for an excellent review of phase transfer catalysis.) The mild reaction

conditions and the volatile nature of the solvents used ensure simplicity of work-up, as emphasized by Cook et al. [14] in analogous work on the synthesis of aliphatic nitriles. Complete removal of acetonitrile from the extraction phase under reduced pressure (30 mm) is also facilitated without employing high temperatures.

The purification of the dinitrile from unreacted dihaloalkane is considered unnecessary as this does not interfere with the preparation of the diimidoester dihydrochloride and is removed in the final washing procedure to yield a pure product. The only drawback of the procedure is the long (40 h) reaction time in the preparation of the dinitrile at room temperature – a minor inconvenience since the procedure needs no attention at this stage. Moreover, the cost of using 18-crown-6 ether is minimal in comparison to the cost of $K^{14}CN$.

Comparison of the crosslinking ability of [^{14}C]-dimethylsuberimidate with that of non-radioactive dimethylsuberimidate using catalase as substrate demonstrated identical reactivity towards the enzyme (fig.1), and fluorography of the gel showed that radioactivity was incorporated into each of the bands. The crosslinking profile was also identical to that obtained using commercially available dimethylsuberimidate. This therefore demonstrates the reliability of the crown ether synthesis in the preparation of [^{14}C]-dimethylsuberimidate and there is no reason why such a synthesis should not be safely adapted to produce [^{14}C]diimidoesters of a very high specific activity. This would facilitate detection of crosslinked species when there is a limited amount of protein available to study, or the interpretation of complex maps derived from electrophoresis of, for example, crosslinked ribosomal proteins.

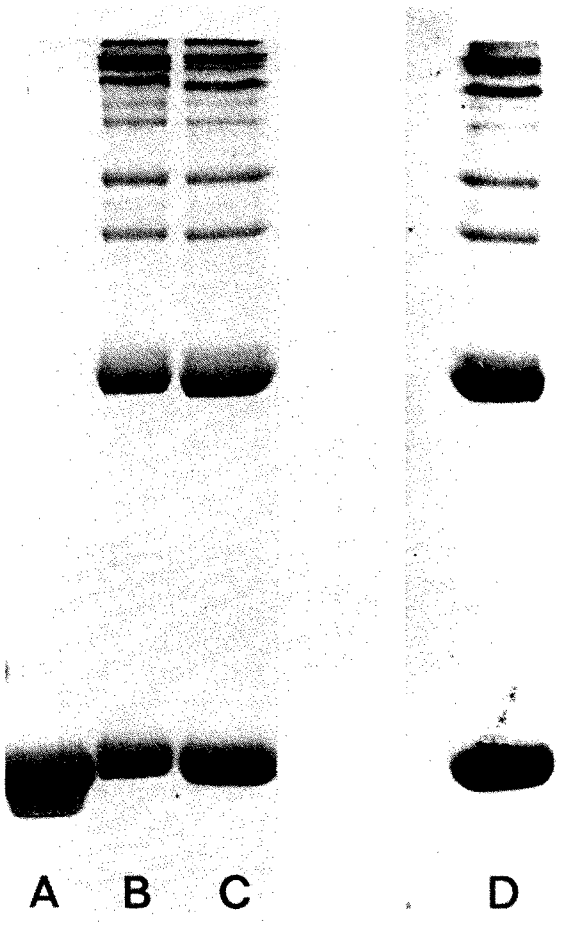

Fig.1. Polyacrylamide slab–gel electrophoresis, in the presence of 0.1% (w/v) SDS, of (A) untreated catalase, (B) catalase crosslinked with 20 mM dimethylsuberimidate, (C) catalase crosslinked with 20 mM [^{14}C]dimethylsuberimidate. The fluorographic detection of radioactivity in track (C) yielded the mirror-image autoradiogram shown in track (D). The observed crosslinking pattern is similar qualitatively with that observed [4], although species greater than the tetramer were unresolved in [4]. The gel presented here clearly resolves higher multimers. The highest order observed, as determined by a plot of log molecular weight against mobility using standard protein markers, is the decamer.

Acknowledgements

Our grateful thanks to Professor W. V. Shaw for helpful discussions. L.P. was the holder of a Research Studentship from the Medical Research Council of Great Britain. This work was supported by a Project Grant of the Medical Research Council of Great Britain to W. V. Shaw.

References

[1] Davies, G. E. and Stark, G. R. (1970) Proc. Natl. Acad. Sci. US 66, 651–656.
[2] Fasold, H., Klappenberger, J., Mayer, C. and Remold, H. (1971) Angew. Chem. Int. edn 10, 795.

[3] Sommer, A. and Traut, R. R. (1975) J. Mol. Biol. 97, 471–481.
[4] Hucho, F., Müllner, H. and Sund, H. (1975) Eur. J. Biochem. 59, 79–87.
[5] Hartman, F. C. and Wold, F. (1967) Biochemistry 6, 2439–2448.
[6] Rasched, R., Bohn, A. and Sund, H. (1977) Eur. J. Biochem. 74, 365–377.
[7] Hartman, F. C. and Wold, F. (1966) J. Am. Chem. Soc. 88, 3890.
[8] Smiley, R. A. and Arnold, C. (1960) J. Org. Chem. 25, 257–258.
[9] Brändström, A. (1956) Acta Chem. Scand. 10, 1197–1198.
[10] Laemmli, U. K. (1970) Nature 227, 680–685.
[11] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[12] Pederson, C. J. (1967) J. Am. Chem. Soc. 89, 7017–7036.
[13] Dehmlow, E. V. (1977) Angew. Chem. Int. edn 16, 493–505.
[14] Cook, F. L., Bowers, C. W. and Liotta, C. L. (1974) J. Org. Chem. 39, 3416–3418.