4-Pyridylmethyl, a Thiol-Protecting Group Suitable for the Partial Synthesis of Proteins

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Cysteine is converted into *S*-4-pyridylmethylcysteine [Gosden, Stevenson & Young (1972) *J. Chem. Soc. Chem. Commun.* 1123–1124] by 4-pyridylmethyl chloride in aqueous propanol at pH 8.3. The derivative is stable to the conditions of total acid hydrolysis. Reduction and alkylation of bovine insulin (pH 8.3, aq. 50% propanol) gives fully *S*-substituted derivatives in excellent yields. The *S*-pyridylmethylated A- and B-chains of insulin were separated by gel filtration: each of them has good solubility properties. The pyridylmethyl group is cleaved by electrolysis in a dilute acid medium, pH 2.6, to give reduced chains. They can be recombined to give insulin in the same yield and with the same degree of biological activity as chains which had not been subjected to the protection and de-protection steps. The results indicate that pyridylmethyl satisfactorily meets requirements for partial synthesis and suggest that it warrants more general use.

In principle, the partial synthesis of proteins offers a means to prepare analogues of a protein which entails carrying out the operations of synthesis on a naturally occurring protein or fragments derived from it (see, e.g., Rees & Offord, 1976). As with other, more conventional, approaches to the synthesis of polypeptides, the aggregate of synthetic steps mandatorily requires the protection of thiol groups. In most instances, this requirement extends to disulphide groups as well, since the disulphide bond has usually been considered to be an insufficient form of protection to presently available synthetic procedures (but see Sieber *et al.*, 1978). In addition, the positions in the protein chain of the two cysteine residues participating in a given disulphide bond are often such that reduction of the disulphide is necessary if the aim of partial synthesis is to be achieved.

Necessary considerations affecting the choice of any protecting group are ease and selectivity of introduction and removal, and stability to the manipulations involved in synthesis. There is a further property which is always desirable and which, at times, becomes essential: a protecting group should confer

good solubility properties on the polypeptide chain to which it is attached. For most large polypeptides this is almost certain to mean good solubility in a milieu which is polar and also at least partially aqueous. Although the introduction of polar groups has been suggested (Wünsch, 1971) and an authoritative review has recently signalled a need for them (Kenner, 1977), their importance has hitherto been insufficiently emphasized.

Many thiol-protecting groups have been used in peptide chemistry, the most widely used being the benzyl group (Sifferd & du Vigneaud, 1935) and certain derivatives of it (Wolman, 1974). Such *S*-protecting groups have been used primarily for the synthesis of short peptides in organic solvents and they are all hydrophobic. Although they can be cleaved with varying degrees of ease, their hydrophobicity is a great disadvantage for partial synthesis. The introduction of the acetamidomethyl group (Veber *et al.*, 1968) provided a means for *S*-protection which, among other advantages, considerably enhanced the solubility of the protected peptide in polar solvents. Its subsequent use in the reversible protection of ribonuclease S protein (Veber *et al.*, 1972) appeared to demonstrate its great promise in meeting the requirements set out above. However, we have shown that the reagent acetamidomethanol modifies tyrosine as well as cysteine residues when it is allowed to react with either a synthetic mixture of the two amino acids or reduced insulin (Seely *et al.*, 1973). Although it has been shown that this side reaction can be circumvented in the case of reduced insulin (Hiskey *et al.*, 1975), the procedure is laborious

Abbreviations used: A, A-chain of insulin without S atoms at positions 6, 7, 11 and 20; B, B-chain without S atoms at positions 7 and 19; Pym, 4-pyridylmethyl; Aet, 2-aminoethyl; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

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and does not overcome the limitation to the general usefulness of this protecting group for partial synthesis.

Gosden *et al.* (1972) have demonstrated that the 4-pyridylmethyl group can be used to protect the thiol group of cysteine. It was found that the protecting group could be removed by mild reductive means such as electrolysis, but was very stable to acidolytic cleavage. Moreover, the presence of the pyridyl nitrogen may also be expected to lead to enhanced solubility of the protected peptide in polar media, particularly in acidic aqueous solvents. Thus the group would appear to be a suitable candidate for thiol protection in partial synthesis: this paper presents our investigation of its usefulness.

Experimental

Materials

Acetic acid and mercury were obtained from Siegfried A.G., Zofingen, Switzerland; the former was redistilled before use, the latter was washed with 20% HNO_3 and water. Most of the other chemicals and solvents were obtained from Fluka A.G., Buchs, Switzerland, and used without purification. Tributylphosphine was kept as a 1–5% solution in propanol under N_2 ; the phosphine content of the solution was determined iodometrically (Rüegg & Rudinger, 1974a, 1977) shortly before use. Propan-1-ol, referred to simply as propanol, was the standard co-solvent. 4-Pyridylmethyl chloride hydrochloride was obtained from BDH Chemicals, Poole, Dorset, U.K. For purification, the commercial material (3.8 g) was dissolved in 100 ml of 1 M- NaHCO_3 /ethyl acetate (1:1, v/v) and the aqueous layer was adjusted to pH 9 with 1 M- NaOH . The mixture was shaken and the organic phase was separated and washed twice with 30 ml of water. The stable hydrochloride was regenerated by extracting the organic phase with 25 ml of 1 M- HCl and twice with 20 ml of water. The combined aqueous extracts were evaporated to dryness by flash evaporation and dried over solid KOH . The product was recrystallized from 20 ml of hot ethanol to give 2.9 g; the melting point was 173–174°C.

The Sephadex cross-linked dextran gels (Pharmacia AB, Uppsala, Sweden), CM-cellulose (CM-52; Whatman, Maidstone, U.K.) and Dowex resin (Dow Chemical Co., Midland, MI, U.S.A.) were prepared for use in accordance with the makers' instructions.

Bovine insulin was a gift from Farbwerke Hoechst A.G., Frankfurt/Main, Germany, obtained through the courtesy of Dr. R. Geiger, and from the Eli Lilly Research Laboratories, Indianapolis, IN, U.S.A., by courtesy of Dr. J. E. Shields. The material was freed of zinc and proinsulin by gel filtration through Sephadex G-50 (fine) (Steiner & Oyer, 1967) in 50% acetic acid (Rüegg & Rudinger, 1974b) and recovered

by freeze-drying. The protein content of the samples was determined spectrophotometrically by using $\epsilon_{280} = 5520 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for insulin in 35 mM- HCl (Praisman & Rupley, 1968).

Methods

Optical rotations were measured with a Perkin-Elmer model 141 photoelectric polarimeter. All evaporations were carried out under reduced pressure in a rotary evaporator at bath temperatures not exceeding 35°C.

Thin-layer electrophoresis. This was carried out in a moist-chamber apparatus (Camag A.G., Muttenz, Switzerland). For amino acids, a potential gradient of about 25 V/cm for 45–90 min was used, the support was cellulose on glass (precoated t.l.c. plates, 0.1 mm; Merck, Darmstadt, Germany), the electrolytes were formic acid/acetic acid/water (1:1:8, by vol.) (pH 1.6) and acetic acid/pyridine/water (6:23:970, by vol.) (pH 5.6) and detection was by ninhydrin. For the modified insulin chains (samples 10–50 nmol in 5–10 μl), a potential gradient of 10–15 V/cm was applied for 2 h, the support was cellulose acetate (CA-electrophoresis strips; Schleicher und Schüll, Dassel, Germany), the electrolyte was 2.4 M-formic acid in 7 M-urea (see, e.g., Krail *et al.*, 1971); the strips were stained for 15 min with a 0.2% solution of Ponceau S in 0.1 M-acetic acid.

Isoelectric focusing. Isoelectric focusing of S-pyridylmethylated chains (20–60 nmol) was carried out in 7% polyacrylamide gels (0.6 cm $\times$ 7 cm). The pH gradient was established with Ampholines of pH 3–10 (LKB-Produkter, Bromma, Sweden) and a potential of 60 V during 3.75 h (Wrigley, 1968).

Desalting by gel filtration. Protein fractions were freed of salts and urea by gel filtration through a column (2.6 cm $\times$ 35 cm) of Sephadex G-25 (medium) in 50% acetic acid or in 0.01 M- NH_4HCO_3 , pH 8.2, at flow rates of 30–50 ml/h. Elution was monitored by absorbance at 280 nm; when necessary, pooled fractions were evaporated under reduced pressure, with several additions of water to remove the volatile buffer, before being freeze-dried.

Amino acid analyses. Samples were hydrolysed with constant-boiling HCl at 110°C for 22 h in sealed evacuated tubes and analysed on a Beckman model 120B amino acid analyser under standard conditions (Spackman, 1967). Values are normalized to the sum of alanine and glycine, but are not corrected for incomplete hydrolysis (e.g. the isoleucine–valine bond in the A-chain) or for partial decomposition (serine, threonine, tyrosine) (Moore & Stein, 1963). The analysis for unmodified insulin is given in Table 1 (column 1) for reference. S-4-Pyridylmethylcysteine was eluted with histidine from the long column of the amino acid analyser (Spackman, 1967) and the colour constant was determined to be 0.85 times that for

Table 1. *Amino acid analysis*
The value for histidine includes values for S-4-pyridylmethyleysteine. The value for proline includes values for cysteine.

Material	...	Number	Composition (residues/peptide)										Insulin reference	Insulin from Pym-chains
			A(SPym) ₄ B(SPym) ₂	B(SPym) ₂	A(SPym) ₄	B(SSO ₃ H) ₂ 'native'	B(SSO ₃ H) ₂ from 'native'	A(SAet) ₄ from 'native'	A(SSO ₃ H) ₄ crude from A(SPym) ₄	A(SSO ₃ H) ₄ purified from A(SPym) ₄	A(SSO ₃ H) ₄ from Pym-chains			
Lys		1	1.01	1.02	1.00	1.00	0.99	0	0	0	0	11	1.00	—
Cys(Aet)		—	—	—	—	—	—	3.60	—	—	—	—	—	—
His		1.91	6.88	3.69	1.93	1.97	1.97	0	0	0.21	0	1.89	0	1.89
NH ₃		5.99	6.08	3.72	2.50	4.80	5.74	3.49	4.10	4.10	3.82	7.31	3.82	7.31
Arg		0.97	0.99	0	0.97	1.00	0	0	0	0	0	0.98	0	0.98
Asp		2.97	2.98	2.00	1.04	1.01	2.08	2.04	2.03	2.03	1.96	3.03	1.96	3.03
Thr		0.95	0.97	0	0.97	0.96	0	0	0	0	0	0.96	0	0.96
Ser		2.69	2.68	1.88	0.94	0.91	1.93	1.67	1.90	1.90	1.76	2.83	1.76	2.83
Glu		7.04	7.05	4.07	3.10	3.03	4.09	4.09	4.02	4.02	4.04	7.03	4.04	7.03
Pro		1.03	1.05	0	1.48	1.11	0	0.80	0.53	0.53	1.02	1.11	1.02	1.11
Gly		4.04	4.01	3.00	3.04	2.98	1.01	0.98	0.99	0.99	0.98	3.91	0.98	3.91
Ala		2.96	2.99	2.00	1.96	2.02	0.99	1.02	1.01	1.01	1.02	3.09	1.02	3.09
†(Cys) ₂		5.54	0	0	0.22	0.41	0	0.44	1.10	1.10	0.63	4.73	0.63	4.73
Val		4.54	4.74	1.63	2.68	2.95	1.69	1.66	1.54	1.54	1.77	4.51	1.77	4.51
Ile		0.68	0.78	0.58	0	0	0.61	0.64	0.65	0.65	0.69	0.79	0.69	0.79
Leu		6.05	6.08	4.05	4.04	4.01	2.03	1.97	2.05	2.05	2.03	5.99	2.03	5.99
Tyr		3.80	3.83	1.97	1.92	1.79	1.96	1.80	1.80	1.80	1.98	3.82	1.98	3.82
Phe		2.92	2.96	2.96	2.84	2.90	0	0	0	0	0	2.81	0	2.81

lysine. Even though no losses were found when free pyridylmethylcysteine alone was subjected to the conditions of acid hydrolysis, values for this derivative in a total hydrolysate were always about 10% too low (see Table 1).

Electrolytic reductions. These were carried out in a simple apparatus similar to that described by Scopes *et al.* (1965). A glass cylinder (3.5 cm × 7 cm) containing a spiral of tungsten wire submerged in mercury served as cathode compartment. The solution to be electrolysed was layered on top of the mercury, together with a magnetic stirring bar. A spiral of platinum wire in a glass tube (2.7 cm internal diam.) fitted with a porous glass disc (D4) at one end served as anode compartment. All reductions were done at 6 V at room temperature and a slow stream of N₂ was blown gently into the cathode compartment.

In time-course experiments, free thiol groups were determined with Nbs₂ (Ellman, 1959). In the case of the insulin chains, this determination had to be done in 1 M-Tris/HCl/6 M-urea, pH 8, to prevent precipitation of the reduced insulin chains.

S-4-Pyridylmethyl-L-cysteine

L-Cysteine hydrochloride (1.58 g; 10 mmol) in 1 M-NaHCO₃ (30 ml) was treated, under N₂, with 4-pyridylmethyl chloride hydrochloride (1.64 g; 10 mmol) in 0.5 M-NaHCO₃/propanol (1:1, v/v) (20 ml). The pH was maintained at 8.3 by the periodic addition of 6 M-NaOH. After 12 h at room temperature, the solution was brought to pH 2 with concentrated HCl and applied to a column (2.7 cm × 35 cm) of Dowex 50W (X8; H⁺ form; 20–50 mesh). After the column had been washed with water, the product was eluted with aq. 1 M-NH₃ (200 ml) and the eluate was evaporated to dryness on a flash evaporator. The crude material was taken up in boiling water (50 ml). The solution was filtered to remove traces of cystine, diluted with hot propan-2-ol (60 ml) and left to reach room temperature. The crystalline product was collected by filtration, washed with propan-2-ol and dried. Yield: 1.84 g (87%); m.p. 219–221°C (decomp.); $[\alpha]_D^{25} -11.5^\circ$ (c. 2.4 in 5 M-HCl), light-absorption maxima in 1 M-HCl, 217 and 256 nm (ϵ 8500 and 4790 M⁻¹ · cm⁻¹ respectively), minimum 236 nm (ϵ 2220 M⁻¹ · cm⁻¹) (Found: C, 50.6; H, 5.6; N, 13.1; C₉H₁₂N₂O₂S requires C, 50.9; H, 5.7; N, 13.2%). Literature values (Gosden *et al.*, 1972): m.p. 210–211°C, $[\alpha]_D^{20} -10^\circ$ (c. 1 in 1 M-HCl).

Reduction and S-pyridylmethylation of insulin

Insulin (574 mg; 100 μmol) in 0.5 M-NaHCO₃/propanol (1:1, v/v) (100 ml) was treated with tributylphosphine (360 μmol in 9.6 ml of propanol) and 4-pyridylmethyl chloride hydrochloride (118 mg; 720 μmol, dissolved in 10 ml of water and neutralized with 0.36 ml of 2 M-NaOH). After 16 h at room temperature, the solution was concentrated to 50 ml by

flash evaporation and adjusted to pH 5.0 with acetic acid. The solution was placed in the 100 ml beaker of a Hollow Fiber Dialyser b/HFD-1 (Dow) and dialysed against demineralized water (12 litres) for 3 h. The dialysis residue was evaporated and finally freeze-dried to give 580 mg (92%) of a mixture of A- and B-chain derivatives. Amino acid analysis is given in Table 1 (column 2).

Separation of S-pyridylmethylated A- and B-chains

A portion (536 mg) of the above chain mixture was dissolved in 50% acetic acid (20 ml) and applied to a column (5.1 cm × 109 cm) of Sephadex G-50 (fine) which had been equilibrated with 50% acetic acid. Elution was carried out with the same solvent at a flow rate of 110 ml/h. Fractions were pooled, concentrated to about 10 ml and rechromatographed on the same column. The main peaks were isolated and freeze-dried to give 295 mg (96%) of B(SPym)₂ and 202 mg (88%) of A(SPym)₄. Amino acid analyses are given in Table 1 (columns 3 and 4).

Electrolytic reduction of S-4-pyridylmethylcysteine: time-course experiment

In a typical experiment, S-4-pyridylmethylcysteine (1.1 mg; 5 μmol) in 0.025 M-H₂SO₄ (10 ml) was layered on top of the mercury cathode and a potential of 6 V was applied. During the course of the reduction, samples (200 μl) were taken at intervals for the determination of thiol with Nbs₂. For quantitative analysis, ethyleneimine (5 μl) and 0.2 M-sodium borate buffer (1 ml, pH 8.0) were mixed with the sample under N₂ (Cole, 1967) and S-aminoethylcysteine was determined after 15 min reaction time by amino acid analysis.

Electrolytic reduction of B(SPym)₂ and its conversion into the S-sulpho derivative

B(SPym)₂ (70.4 mg; 19 μmol) was dissolved in 0.025 M-H₂SO₄/6 M-urea (pH 2.6, 10 ml) and electrolysed for 90 min. The clear cathode solution was transferred into a flask and 1 M-Tris/HCl, pH 8.5 (1 ml), sodium tetrathionate (0.5 g), sodium sulphite (1 g) and guanidine hydrochloride (12.4 g) were added; the solution was adjusted to pH 8.5 by the addition of aq. 4 M-NH₃. After 21 h at room temperature, acetic acid (5 ml) was added and the solution was desalted by gel filtration in 50% acetic acid, evaporated and freeze-dried to yield 63.2 mg (93%). This material was purified on a column (2.6 cm × 56 cm) of CM-cellulose (Whatman CM-52). Elution at 33 ml/h was performed with 0.04 M-sodium acetate/8 M-urea (adjusted to pH 4.0 with acetic acid). Fractions were pooled as indicated in Fig. 2(b), desalted by gel filtration in 50% acetic acid, evaporated and freeze-dried to give purified B(SSO₃H)₂ (43.9 mg, 70%). On electrophoresis at pH 2.2 this material showed one

band with the same mobility as an authentic sample. For amino acid analysis, see Table 1 (column 6).

Electrolytic reduction of A(SPym)₄ and its conversion into A(SAet)₄

In a time-course experiment, A(SPym)₄ (6.6mg; 2.3μmol) was dissolved in 0.1M-H₂SO₄/4M-urea, pH1.9 (10ml), and electrolysis was carried out for 60min. The catholyte was adjusted to pH8.5 with 1M-NaOH, and propanol (8ml) containing tributylphosphine (30μmol) as oxygen scavenger and ethylenimine (100μl; 1.9mmol) were added (Cole, 1967). After 2h the solution was desalted by gel filtration in 0.01M-NH₄HCO₃. The protein-containing fractions were pooled, concentrated and freeze-dried to yield 4.3mg of A(SAet)₄. For amino acid analysis see Table 1 (column 7).

Electrolytic reduction of A(SPym)₄ and its conversion into A(SSO₃H)₄

In a preparative experiment, A(SPym)₄ (42.2mg; 14.2μmol) was dissolved in 0.025M-H₂SO₄/6M-urea, pH2.6 (10ml), and reduction was carried out for 90min. After about 20min, slight precipitation began and continued for the rest of the experiment. The mixture was transferred to a beaker and was converted into A(SSO₃H)₄ in a manner quite analogous to that described above for the preparation of B(SSO₃H)₂. After desalting in 50% acetic acid and freeze-drying, 37.8mg (98%) of product was obtained. This material was purified by chromatography on CM-cellulose (Fig. 2a) by the procedure detailed above for the purification of B(SSO₃H)₂ and 30.1mg (80%) of A(SSO₃H)₄ was obtained. On electrophoresis at pH2.2 this material showed the same three bands as a sample of A(SSO₃H)₄ prepared directly from insulin: a major one and two minor ones having $m_{A(SSO_3H)_4}$ of 0.71 and 0.43. For amino acid analysis, see Table 1 (column 10).

Chain recombination and crystallization of insulin

A(SSO₃H)₄ (67mg; 25μmol) and B(SSO₃H)₂ (89mg; 25μmol), obtained either by direct sulphytolysis of insulin (Katsoyannis *et al.*, 1967) or after removal of the 4-pyridylmethyl protecting groups, were recombined and the insulin formed was purified by gel filtration and chromatography on CM-cellulose as previously described (Rüegg & Gattner, 1975). Insulin was crystallized from citrate buffer containing 16% acetone as described by Kung *et al.* (1966).

Bioassay

The glycolytic activity of the insulin preparations was measured by means of the isolated rat epididymal fat-pad assay described by Froesch *et al.* (1963).

Results

Introduction of the pyridylmethyl group

4-Pyridylmethyl chloride was found to alkylate the thiol group of cysteine readily in aqueous solution at room temperature and at about pH8, and S-4-pyridylmethylcysteine was isolated in good yield. Obviously, S-pyridylmethylation of insulin requires prior reduction of the three disulphide bonds. In earlier work (Rüegg & Rudinger, 1974a, 1977) we had found that tributylphosphine is an excellent reagent for the reduction of the disulphide bonds of proteins and so it was used for that purpose throughout the work reported here. Since the phosphine and its oxide do not react with pyridylmethyl chloride under the conditions used, both reagents were added together.

When insulin was treated at pH8.3 during 16h with only a 20% excess of each of the reducing and alkylating agents, the mixture of S-pyridylmethylated A- and B-chains was obtained, after dialysis and freeze-drying, in good yield. Amino acid analysis of this 1:1 chain mixture indicated that reduction and S-alkylation was complete (Table 1, column 2). Separation of the alkylated chains was most conveniently achieved by gel filtration through Sephadex G-50 or G-75. Although the latter gave slightly improved resolution, the former was preferred for large-scale preparations on account of its higher rigidity. Rechromatography of each of the partially resolved peaks gave each derivative in about 90% yield with less than 1% cross-contamination (see amino acid analyses: Table 1, columns 3 and 4). Electrophoresis on cellulose acetate at pH2.2 revealed a single band for each product having the same mobility as the corresponding S-aminoethyl derivative (Rüegg & Rudinger, 1974a). Likewise, isoelectric focusing gave a sharp precipitation line for each material.

Removal of the 4-pyridylmethyl group

Although the pyridylmethyl group can be removed by sodium in liquid NH₃, electrolytic cleavage by the method described by Young and his colleagues (Scopes *et al.*, 1965; Gosden *et al.*, 1972) is milder and much to be preferred. The time course of the liberation of thiol groups from S-pyridylmethylcysteine was followed colorimetrically by means of Nbs₂ (Ellman, 1959) as well as by formation of the S-aminoethylcysteine derivative, which was then determined by amino acid analysis. These experiments indicated that electrolysis proceeded rapidly and followed an exponential function. They showed that increasing the peptide concentration 10-fold or decreasing the acidity by the same factor led to a halving of the rate of reduction.

The attempt to apply the data obtained with *S*-pyridylmethylcysteine to the reduction of the pyridylmethylated chains of insulin was hampered by the poor solubility of the reduced chains. The results of time-course experiments with the *SS'*-bispyridylmethylated B-chain are shown in Fig. 1. Only at relatively high acidity and low concentrations of peptide was the rate of reduction similar to that found for the cysteine derivative and did the dithiol product remain in solution (curve 1). At higher concentrations of peptide or lower acidity, additives were needed to prevent precipitation of the reduced chain and this led to a concomitant increase in the time needed for reduction. For preparative purposes, a 1.9 mM solution of peptide in 6M-urea at pH 2.6 was used (curve 5) and the time for reduction was 90 min.

The time course of the reduction of the *SS'S'S''S'''*-tetrakispyridylmethylated A-chain was found to be similar to that of the B-chain derivative, but, owing to the even poorer solubility of the reduced A-chain, urea had to be added at a much lower (0.25 mM) peptide concentration. In a preparative experiment, a 1 mM solution of A(SPym)₄ was electrolysed in 6M-urea at pH 2.6 for 90 min. Under these conditions, a small amount of precipitate began to form after about 20 min and amino acid analysis (Table 1, column 9) of the product revealed the presence of an amount of *S*-4-pyridylmethylcysteine which reflected an approximately 5% contamination with an unreduced or partially reduced derivative. This contaminant could easily be removed at the next stage (Fig. 2a). Further-

more, when a 0.25 mM solution of A(SPym)₄ was electrolysed in 0.1M-H₂SO₄/6M-urea, conditions under which precipitation did not occur, and the product was aminoethylated, the amino acid composition of the product showed no *S*-4-pyridylmethylcysteine. This suggests that incomplete reduction in the preparative experiment was due to occlusion of incompletely reduced material in the precipitate rather than the failure of reduction as such. Moreover, the value for alanine, the product of a possible desulphurization reaction (Katsoyannis, 1964; Shimonishi *et al.*, 1969), was not increased (Table 1, column 7). The reduced chains so obtained were converted into their *S*-sulpho derivatives by oxidative sulphytolysis (Bailey & Cole, 1959; Katsoyannis *et al.*, 1967) and the same experiment was carried out in tandem on insulin. It was necessary to add guanidine hydrochloride to the urea solutions of peptide in order to prevent precipitation, and NH₃ was added as a scavenger for cyanate (Stark *et al.*, 1960). The *S*-sulpho chains were purified by chromatography on CM-cellulose (Katsoyannis *et al.*, 1967). Since the three minor peaks preceding the main peak in the chromatogram of the B-chain derivative (Fig. 2b) appeared in the same positions and proportions in the preparation obtained by direct sulphytolysis of insulin, they are unlikely to be derived from electrolysis and are probably B-chain oligomers. Electrophoresis of material obtained from the main peak revealed the presence of only one band in both preparations. The amino acid analysis (Table 1,

Fig. 1. Time course of the electrolysis of B(SPym)₂. Reductions were performed in 10 ml of solvent at 6 V and thiol liberated was analysed with Nbs₂. Curve 1, 0.24 mM-B(SPym)₂ in 0.05 M-H₂SO₄; curve 2, 0.25 mM-B(SPym)₂ in 0.05 M-H₂SO₄ in 50% dimethylformamide; curve 3, 0.25 mM-B(SPym)₂ in 0.025 M-H₂SO₄ in 6M-urea; curve 4, 0.25 mM-B(SPym)₂ in 0.005 M-H₂SO₄ in 50% dimethylformamide; curve 5, 1.90 mM-B(SPym)₂ in 0.025 M-H₂SO₄ in 6M-urea.

Fig. 2. Purification of the *S*-sulpho chains of insulin. Chromatography of the *S*-sulpho chains obtained after electrolytic removal of *S*-4-pyridylmethyl protecting groups and sulphytolysis was carried out on a CM-cellulose column (2.6 cm × 56 cm) in 0.04 M-sodium acetate/8M-urea, pH 4.0. (a) A(SSO₃H)₄; (b) B(SSO₃H)₂. Horizontal lines indicate pooling of fractions.

column 6) of this material derived from $B(\text{SPym})_2$ was in good agreement with that for authentic $B(\text{SSO}_3\text{H})_2$ (Table 1, column 5). The main peak of the material obtained from electrolysis/sulphitolysis of $A(\text{SPym})_4$ was eluted at the same position as authentic material (Fig. 2a). The 5% impurity mentioned above was separated as a small shoulder. The amino acid composition of the purified $A(\text{SSO}_3\text{H})_4$ showed no residual *S*-4-pyridylmethylcysteine (Table 1, column 10) and was in good agreement with that of the reference material (Table 1, column 8). On electrophoresis, both preparations behaved identically and gave rise to one major band and two, more slowly moving, minor ones. The latter are probably incompletely sulphitolysed A-chains (Zahn & Drechsel, 1968). They can be removed (Rüegg & Gattner, 1975), but this is not essential since the preparation is to be used directly for chain recombination.

Resynthesis of insulin

The *S*-sulpho chains obtained after de-pyridylmethylation and oxidative sulphitolysis were combined in a 1:1 molar ratio for the re-synthesis of insulin. A control experiment with *S*-sulpho chains obtained directly from insulin was run in tandem.

Chain recombination and successive fractionation of the products on Sephadex G-50 and CM-cellulose were carried out as described by Rüegg & Gattner (1975). The final chromatographic step is shown in Fig. 3. After desalting, 13.1 mg (9.1% overall re-synthesis yield) of insulin was obtained from the de-pyridylmethylated chains and 12.5 mg (8.7%) in the control experiment. The material obtained from peak B had a similar amino acid composition to that of insulin and may be a deaminated product. The insulin fractions from both experiments were analysed further: the amino acid composition of the test sample (Table 1, column 11) showed no significant deviation from that of the control or of native insulin (column 1). Both materials gave one band in the expected position in electrophoresis at pH 2.2 and 4.8.

The biological activity of the insulin derived from the de-protected chains was 17 ± 6 units/mg compared with 19 ± 6 units/mg for the control. Both materials could be crystallized from citrate buffer containing zinc, to give the rhombohedral crystals characteristic of zinc-insulin.

Discussion

The results clearly indicate that 4-pyridylmethyl chloride reacts smoothly and selectively with both cysteine and the reduced insulin chains to give the corresponding *S*-alkylated derivatives in excellent yield and a high state of purity. From a practical point of view it is convenient that the compatibility of the reagents permits the successive reactions of reduction and alkylation to be carried out in a single operation. *S*-Pyridylmethylcysteine is quite water-soluble and completely stable to the conditions of acid hydrolysis of proteins. The protecting group also confers good solubility properties on the modified insulin chains, making them easy to handle in the partially aqueous media used in this study. Finally, electrolysis permits the protecting group to be cleaved cleanly and quantitatively under mild conditions to regenerate reduced insulin chains. Thus, in its solubilizing effect and its susceptibility to cleavage under mild conditions, the pyridylmethyl group certainly possesses two marked advantages compared with the benzyl group (see, e.g., Tsou *et al.*, 1961), the type of *S*-protection most commonly used hitherto for the chains of insulin. The de-protected chains can subsequently be recombined to give insulin in the same yield and with the same degree of biological activity as chains which had not been subjected to the protection and de-protection steps. The fact that insulin activity can be reconstituted to the same degree from both preparations provides the most convincing demonstration that the pyridylmethyl group satisfactorily meets the requirements

Fig. 3. Final step in the purification of resynthesized insulin: comparison of two preparations

Chromatography of material prefractionated by gel filtration was carried out on CM-cellulose in 0.1 M-acetic acid/7 M-urea, pH 3.9 (---, gradient 0–0.1 M-NaCl). Pure insulin is eluted as peak C; the position of the leading shoulder of peak C in (b) corresponds to that of a deaminated product. (a) Material originates directly from insulin; (b) material originates from de-pyridylmethylated chains. Insulin yields were 12.5 mg in (a) and 13.1 mg in (b).

for a protecting group set out in the introduction to this paper and suggests its more general application for thiol protection in partial synthesis.

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