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Reactions of I,I-Diacetoxyiodobenzene with Proteins: Conversion of Amide Side-chains to Amines

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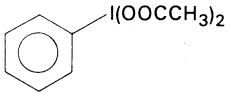
Abstract

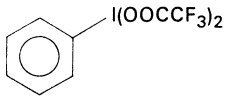
Experiments with the *N*-benzyloxycarbonyl derivatives of asparagine and glutamine as models show that, in unbuffered solutions, I,I-diacetoxyiodobenzene (1) is more effective than the corresponding trifluoroacetoxy derivative (2) for converting the amide side-chains of proteins to amines. Maximum modification of the glutamine residues of insulin and lysozyme occurs within 1–2 h of treatment with 1 in aqueous methyl cyanide at 20°C, but asparagine residues react more slowly. The amide side-chains are converted to the corresponding amines in at least 90% yield, as shown by analysis of acid hydrolysates for aspartic acid, α,β-diaminopropionic acid, glutamic acid and α,γ-diaminobutyric acid. Numerous side-reactions also occur, tyrosine, cystine, methionine, arginine, lysine and *N*-terminal residues all being modified to some extent.

Introduction

I,I-Bis-trifluoroacetoxyiodobenzene (2) was recently recommended, in preference to I,I-diacetoxyiodobenzene (1), for converting primary amides to primary amines (Radhakrishna *et al.* 1979). It has also been used to convert *C*-terminal amide groups to amines, as part of a procedure for the stepwise removal of amino acid residues from the *C*-terminus of several peptides (Parham and Loudon 1978). One of the side-reactions of this procedure is the conversion of side-chain amides to amines, that is, the conversion of asparagine (Asn) residues to α,β-diaminopropionic acid (A_2Pr) residues, and glutamine (Gln) residues to α,γ-diaminobutyric acid (A_2Bu) residues.



(1)



(2)

In the work to be described we have examined the potential of (1) and (2) for selectively modifying the Asn and Gln residues in peptides and proteins. This procedure may be useful for changing the electrophoretic and solubility behaviour of proteins, and for facilitating the identification of Asp, Asn, Glu and Gln residues in peptides. Experiments were carried out with the *N*-benzyloxycarbonyl (Z-) derivatives of Asn and Gln, as well as with the proteins insulin, lysozyme and keratin (wool).

Materials and Methods

Materials

I,I-Diacetoxyiodobenzene (1) (from Aldrich Chemical Co., Milwaukee, Wis.) was purified by recrystallization from glacial acetic acid. Recrystallization from trifluoroacetic acid gave I,I-bis-trifluoroacetoxyiodobenzene (2) (cf. Spyroudis and Varvoglis 1975). Z-Asn and Z-Gln were prepared from the corresponding L-amino acids by reaction with benzyloxycarbonyl chloride in sodium bicarbonate solution, and purified by recrystallization from water and aqueous methanol, respectively.

Z-A_2Bu was prepared by stirring a suspension of Z-Gln (0·56 g) and (1) (0·96 g) in a mixture of methyl cyanide (4 ml) and water (2 ml) for 2 h at room temperature. Ethyl acetate and 2 M hydrochloric acid were added, and the product was then isolated from the aqueous layer by adsorption onto a cation-exchange resin (Zeo-Karb 225, H^+ form), followed by elution with ammonia. Yield 0·31 g, 60%. Addition of ethanol (20 volumes) to an aqueous solution gave colourless needles, m.p. 198°C. Found: C, 57·1; H, 6·3; N, 10·9%. $C_{12}H_{16}N_2O_4$ requires C, 57·1; H, 6·4; N, 11·1%.

A similar procedure was used to prepare Z-A_2Pr from Z-Asn. The product crystallized from water as colourless needles, m.p. 244°C (cf. 243–245°C, found by Moore *et al.* 1976).

Insulin was supplied by Commonwealth Serum Laboratories, Parkville, Vic., and lysozyme by Worthington Chemical Corporation, New Jersey, U.S.A. Merino 64's wool was purified by successive extraction with light petroleum, ethanol and water before use.

Reaction Rates

The rates of formation of amino groups during reaction of Z-Asn, Z-Gln, insulin and lysozyme with (1) or (2) were determined by adding an excess of the reagent in methyl cyanide–water (1 : 1, v/v) to a solution of the substrate in the same solvent mixture at 20°C. Aliquots were removed periodically and stored at −60°C to await analysis. Amino contents were measured by a colorimetric ninhydrin procedure (Maclaren and Milligan 1981) using Z-A_2Pr, Z-A_2Bu, lysozyme and insulin as standards.

Treatment and Hydrolysis of Proteins

A solution of insulin (10 mg) and (1) (20 mg) in 50% (v/v) aqueous methyl cyanide (2·0 ml) was kept for 1·5 h at 20°C and then passed through a Sephadex LH-20 column equilibrated with water–methyl cyanide–acetic acid (50 : 49 : 1 v/v) to isolate the treated protein; about 1 h was required for this separation. In a second experiment insulin was treated with (1) for 20 h under the same conditions and the modified protein was then isolated as described above.

Lysozyme (100 mg) was treated with (1) (250 mg) in 50% (v/v) aqueous methyl cyanide (15 ml) for 1 h, and also for 20 h, and then isolated as above.

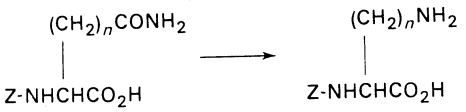
Wool samples (50 mg) were treated at room temperature with (1) or (2) (50 mg) in 50% (v/v) aqueous methyl cyanide (2·5 ml) for 5, 16 and 24 h, and then rinsed several times with methyl cyanide, followed by water.

Samples (5 mg) of the above proteins were hydrolysed with 6 M hydrochloric acid (5 ml) in evacuated sealed tubes at 108°C for 24 h, and analysed on a Beckmann amino acid analyser. The basic amino acids were separated by elution from a column of Hamilton HP-B80 resin with 0·35 M citrate buffer (pH 5·20) at 49°C; this procedure satisfactorily resolves A_2Pr, A_2Bu and the usual basic amino acids. Colour constants for A_2Pr and A_2Bu (relative to lysine) were 0·22 and 0·91, respectively.

Results and Discussion

Preliminary experiments with the model amides Z-Asn and Z-Gln in unbuffered aqueous methyl cyanide showed that I,I-diacetoxyiodobenzene (1) reacts much faster than the corresponding trifluoroacetoxy compound (2) to give the corresponding amines Z-A_2Pr and Z-A_2Bu (see Fig. 1). This result was unexpected, as (2) was recently shown to be superior to (1) for converting amides to amines (Radhakrishna *et al.* 1979). The large difference in reaction rates is probably due to the lower pH of the

reaction mixture when (2) is used rather than (1) (pH 1·5 *v.* 4), since (2) was found to react as fast as (1) if sodium acetate was added to maintain pH 4.



Z-Asn ($n = 1$) Z-A_2Pr ($n = 1$)
Z-Gln ($n = 2$) Z-A_2Bu ($n = 2$)

The conversion of Z-Asn and Z-Gln to the corresponding amines is not quantitative, and levels out at about 85% conversion (see Fig. 1). Apparently Z-A_2Bu

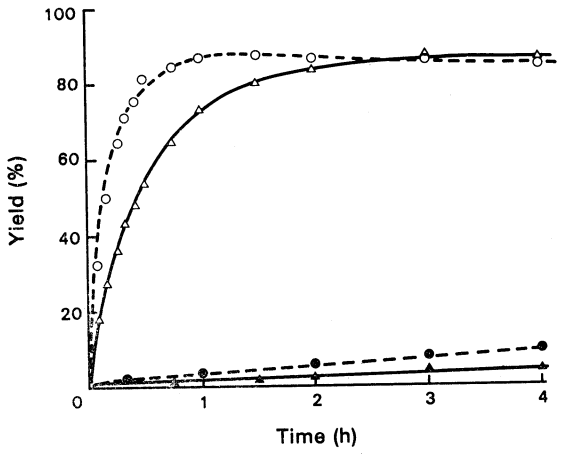
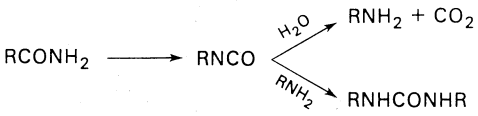
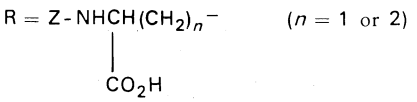


Fig. 1. Rates of conversion of Z-Asn (0·005 M) to Z-A_2Pr with 0·04 M (1) (△) or 0·04 M (2) (▲) and of Z-Gln (0·005 M) to Z-A_2Bu with 0·04 M (1) (○) or 0·04 M (2) (●) at 20°C. Solvent: aqueous methyl cyanide (50% v/v). Yields were determined colorimetrically with ninhydrin.

is slowly degraded by (1), as its concentration reaches a maximum in 1 h, and falls gradually thereafter. The low yields may also be due to reaction between the amines and their precursor isocyanates to give ureas:





Alternatively, cyclization reactions may occur, as Shiba *et al.* (1968) have shown that reaction of Z-Asn with hypobromite, which also proceeds *via* an isocyanate, gives the heterocyclic compound, 1-benzyloxycarbonyl-2-oxoimidazoline-5-carboxylic acid. The same authors have shown that treatment of Z-Gln with sodium hypo-

bromite gives a condensation product of the original amide with the corresponding isocyanate:

$$RCONH_2 + RNCO \rightarrow RCONHCONHR.$$

However, thin-layer chromatography of reaction mixtures of Z-Gln and (1) revealed no trace of this *N*-acylurea.

Reaction rate studies with insulin and lysozyme also show that (1) is preferable to (2) for converting amide side-chains to amines (see Fig. 2). Although (2) reacts much faster than (1) with lysozyme at first, (1) introduces considerably more amino groups than (2) after reaction for 4 h (5·8 *v.* 3·4 residues per mole, assuming that A_2Pr, A_2Bu, lysine and *N*-terminal amino acid *residues* all give the same ninhydrin color yields). The actual rates at which Asn and Gln are modified may be a little higher than indicated in Fig. 2, due to destruction of *N*-terminal residues (see later), as well as lysine, A_2Bu and possibly A_2Pr residues.

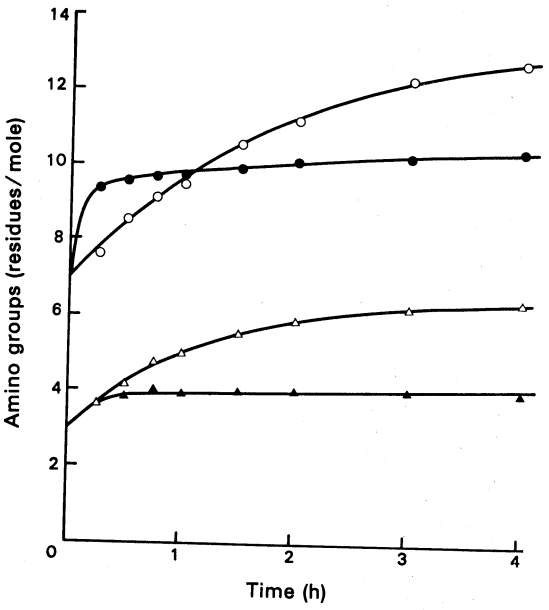


Fig. 2. Rates of incorporation of amino groups into lysozyme (○,●) and insulin (△,▲) during treatment with 0·016 M (1) (open symbols) or (2) (closed symbols) in aqueous methyl cyanide (50% v/v) at 20°C. The protein concentration was 1·6 mg/ml. Yields were determined colorimetrically with ninhydrin.

Analysis of acid hydrolysates of the treated proteins for Asp, Glu, A_2Pr and A_2Bu shows that Asn is converted to A_2Pr, and Gln to A_2Bu in yields averaging over 90%, based on the decrease in Asp and Glu contents (see Tables 1 and 2). Gln residues are modified extensively (*c.* 80%) within 1–2 h, but less than 40% of Asn residues are modified in this time. Prolonged treatment does not substantially change the extent of Gln modification, but Asn modification increases to 72% after 20 h.

Unfortunately, neither (1) nor (2) reacts specifically with the Asn and Gln residues of proteins. Examination of Table 1 shows that (1) modifies tyrosine, cystine, methionine, lysine and arginine residues. Cystine is converted, in part, to cysteic acid, and methionine to its sulfoxide, but the other residues gave only small amounts of unidentified hydrolysis products. There is also circumstantial evidence from Table 1

that (1) modifies the *N*-terminal lysine residue of lysozyme, the *N*-terminal glycine residue of insulin A-chain and the *N*-terminal phenylalanine residue of insulin B-chain. For example, only one of the six lysine residues of lysozyme reacts with (1) in 1 h, further modification of the remainder taking place very slowly. Similar treatment of insulin rapidly modifies 0·6 of its four glycine residues, and one of its three phenylalanine residues, the others being unaffected by prolonged treatment.

Table 1. Changes in amino acid composition of lysozyme and insulin as the result of treatment with I,I-diacetoxyiodobenzene

Amino acid	Theory (residues/mole)	Found (residues/mole)[A] Untreated	1 h[B]	20 h
		Lysozyme		
Asp	8	20·7	16·41	10·40
Asn	13	0	0	0
A_2Pr	—	0	4·41	9·39
Glu	2	4·71	1·97	1·94
Gln	3	0	0	0
A_2Bu	—	0	2·68	2·46
Tyr	3	2·89	0·11	0·15
Lys	6	5·99	4·91	4·51
Arg	11	10·83	10·20	9·95
½Cys	8	—	6·64	0·34
Cys Ac	0	0	0·59	4·45
Gly	12	11·80	12·04	12·02
Phe	3	2·88	2·89	2·80
Met	2	1·88	1·46	0·17
		Insulin		
Asp	0	2·96	1·65	0·55
Asn	3	0	0	0
A_2Pr	—	0	1·03	2·17
Glu	4	6·97	4·40	4·06
Gln	3	0	0	0
A_2Bu	—	0	2·34	2·56
Tyr	4	3·81	0·11	0·19
Lys	1	0·99	0·96	0·87
His	2	1·94	1·87	1·69
½Cys	6	5·58	4·99	4·53
Cys Ac	0	Trace	0·11	0·39
Gly	4	4·13	3·54	3·46
Phe	3	2·99	1·97	1·98

[A] The contents of the amino acids not shown change by less than 5% as the result of treatment.
[B] Treatment period 1·5 h for insulin.

By contrast, the glycine and phenylalanine residues of lysozyme are unaffected by (1), from which we infer that these residues in insulin react only because they occur in *N*-terminal positions.

Wool reacts more slowly than soluble proteins with (1) or (2). As before, (1) is a more effective reagent than (2) for introducing amino groups, and A_2Bu residues are introduced more readily than A_2Pr residues (see Table 2). Tyrosine, cystine,

Table 2. Changes in the amino acid composition of wool as the result of treatment with I,I-diacetoxyiodobenzene (1) and I,I-bistrifluoroacetoxyiodobenzene (2)

Amino acid	Untreated wool	Content (μmol/g dry wool) after treatment with (1)(5 h)	(1)(24 h)	(2)(16 h)
Asp (+Asn)	539	485	451	560
A_2Pr	0	39	64	16
Glu (+Gln)	999	803	727	911
A_2Bu	0	173	227	130

methionine, lysine and arginine modification also occurs. It was hoped that the introduction of additional cationic groups might increase the affinity of the fibre for acid (anionic) dyes, but the yellow/orange colour imparted to the wool by (1) and (2) ruled them out as useful dye-assist reagents.

Acknowledgments

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References

Maclaren, J. A., and Milligan, B. (1981). Analytical techniques. In 'Wool Science: The Chemical Reactivity of the Wool Fibre'. p. 285. (Science Press: Sydney.)

Moore, S., Patel, R. P., Atherton, E., Kondo, M., Meienhofer, J., Blau, L., Bittman, R., and Johnson, R. K. (1976). Synthesis of actinomycin and analogs. 12. Synthesis and some properties and anti-tumor effects of the actinomycin lactam analog, [di-(1′-L-α,β-diaminopropionic acid)]-actinomycin. *J. Med. Chem.* **19**, 766–72.

Parham, M. E., and Loudon, G. M. (1978). Carboxyl-terminal sequential degradation of peptides. *Biochem. Biophys. Res. Commun.* **80**, 1–6.

Radhakrishna, A. S., Parham, M. E., Riggs, R. M., and Loudon, G. M. (1979). New method for direct conversion of amides to amines. *J. Org. Chem.* **44**, 1746–7.

Shiba, T., Koda, A., Kusumoto, S., and Kaneko, T. (1968). Selective chemical cleavage of asparagine peptides. *Bull. Chem. Soc. Japan* **41**, 2748–53.

Spyroudis, S., and Varvoglis, A. (1975). Dehydrogenations with phenyliodine difluoroacetate. *Synthesis* 445–7.

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