Alcoholytic deblocking of N-terminally acetylated peptides and proteins for sequence analysis

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Abstract N-terminal acetylation of polypeptides is a common feature preventing direct Edman degradation. We describe a method for the removal of the acetyl group, with only a low extent of internal peptide bond cleavage, also in large proteins, by treatment at room temperature with trifluoroacetic acid and methanol. The alcohol is essential for selective deacetylation, and it is proposed that the deblocking mechanism consists of an acid-catalyzed nucleophilic substitution involving methanol. The extent of deacetylation is limited, but the initial yield in the sequence analysis can be up to 10%. Deblocking of samples spotted or blotted onto sequencer filters is equally possible as the use of isolated samples from column separations. Deblocking on sequencer filters is also possible directly after negative results on initial sequencer attempts with samples proving to be blocked.

Key words: Deacetylation; N-terminal deblocking; Trifluoroacetic acid; Methanol; Alcoholysis; Edman degradation; Amino acid sequence; Electroblotting

1. Introduction

Sequence analysis of polypeptides by Edman degradation is dependent on a free N-terminal α-amino group. However, many proteins have a blocked N-terminus which prevents the reaction with phenyl isothiocyanate. Most frequently the blocking moiety is an acetyl group [1,2], estimated to occur in 80% of the soluble proteins in mammalian cells [3], and commonly the N-terminal residue is serine (in about 40% of known acetylated proteins [1]). The standard analytical procedure with blocked proteins is enzymatic cleavage, isolation of fragments by reverse-phase HPLC and peptide analysis. Drawbacks with that technique are high protein consumption, long handling times, and maintained inaccessibility of the N-terminal peptide to Edman degradation. To deblock the N-terminal fragment, both enzymatic and chemical protocols have been reported [2,4,5]. Among chemical cleavages an interesting approach employs neat trifluoroacetic acid at elevated temperature (45–65°C) with incubation overnight or for 3 days [5]. However, for large polypeptides and proteins this gives too high a background for clear sequence interpretation [5] because of internal peptide bond cleavage due to low specificity in the deacetylation chemistry employed.

In this report we present a protocol for direct and specific chemical deblocking of N-terminally acetylated polypeptides by treatment with trifluoroacetic acid and methanol, resulting in a very low amount of internal peptide bond cleavage. The specificity is dependent on the presence of methanol and the deacetylation is interpreted to proceed via reactions involving the alcohol. The protocol has been successfully tested on several enzymes (cf. [6–14]) and is found to be efficient for both intact proteins and N-terminal fragments, and for both isolated samples and samples on filters.

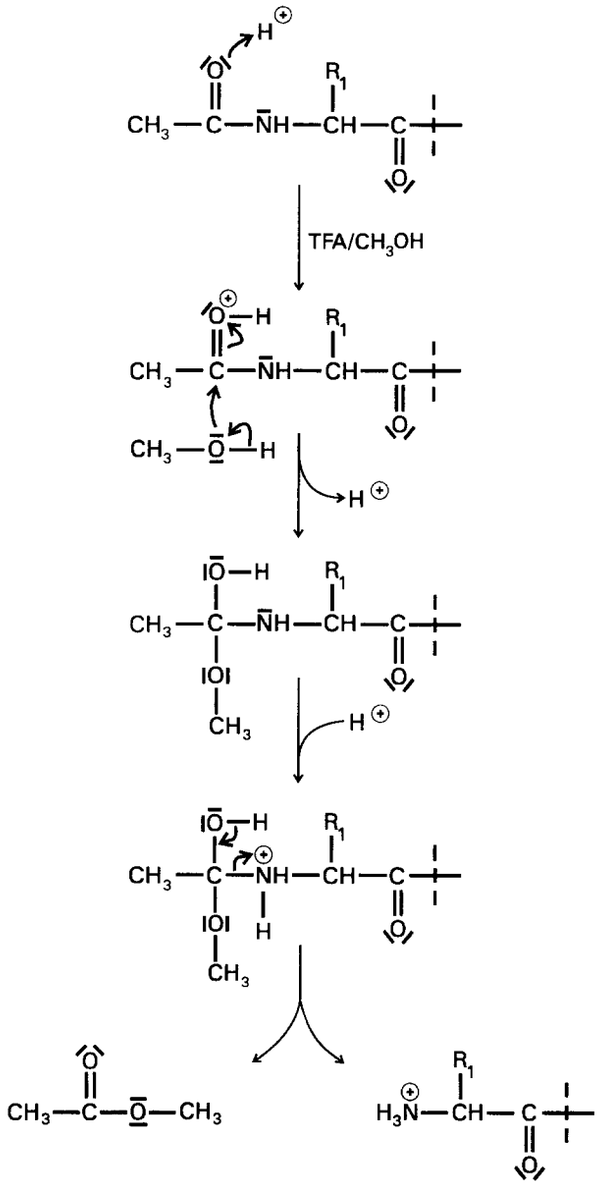

Fig. 1. Suggested mechanism for cleavage of the amide bond during N-terminal deacetylation of polypeptides with TFA and methanol. R_1 is the side-chain of the N-terminal residue.

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2. Materials and methods

The polypeptides employed were obtained from natural sources [6–11] or prepared synthetically [15]. Samples for deacetylation were either recovered in solution from column separations and lyophilized in glass tubes, directly spotted onto sequencer filters, or electroblotted to sequencer filters after SDS-polyacrylamide gel separation [16]. The deblocking solution consisted of a trifluoroacetic acid (TFA)/methanol 1:1 (v/v) mixture, made up freshly. For samples lyophilized in glass tubes, 100 μl deblocking solution was added. After a brief vortex treatment the tubes were closed and incubation started. Spotted or electroblotted samples immobilized to sequencer filters were dried, after which the filters were placed in Eppendorf tubes and about 30 μl deblocking solution was added, just enough to completely wet the filters. Before closing the tubes, an additional volume (70 μl) was added at the bottom of each tube, without liquid contact with the filter, to prevent drying and to maintain a saturated atmosphere of TFA and methanol. Incubation was performed at room temperature for 16 h, after which the reagents were removed under vacuum. Following this treatment, the samples were applied to Edman degradation or stored at −20°C until needed. Sequence analysis was carried out with an Applied Biosystems 470A instrument equipped with reverse-phase HPLC for phenylthiohydantoin analysis [16], or with a Milligen Prosequencer 6600 system. Amino acid analysis was performed after hydrolysis for 24 h at 110°C in 6 M HCl/0.5% (w/v) phenol with an Alpha Plus 4151 analyzer (Pharmacia-LKB).

3. Results and discussion

We have tested mixtures of a strong anhydrous acid (TFA) with an alcohol (methanol) for specific deblocking of N-terminally acetylated peptides and proteins before direct application to sequencer degradations. The idea is to promote specific alcoholysis (acid-catalyzed solvolysis, cf. [17]) of the N-terminal acetyl amide bond. The reagents are easily removed by evaporation after the reaction, as is the resulting methyl acetate. In the reaction, TFA is interpreted to act as a catalyst to protonate the carbonyl oxygen of the acetyl group, while methanol is interpreted to play the role of a weak nucleophile, attacking preferentially the acetyl carbonyl carbon, which results in cleavage of the acetyl group (Fig. 1). The hypothetical mechanism is based on considerations of stereochemical constraints, which suggest a greater accessibility of methanol to the acetyl carbonyl carbon at the start of the polypeptide chain than to other amide carbonyl carbons in internal peptide bonds. The latter are adjacent to amino acid side-chains which provide steric hindrance to the alcohol access and attack.

The methodology has been tested on both N-terminal peptides (5–20 residues) and proteins (35–55 kDa or approx. 330–500 residues). The results (Tables 1 and 2) reveal a high specificity but a limited yield, although sequencer initial yields of up to 10% were achieved upon deblocking of polypeptides available in low amounts. The technique has proven equally suitable for lyophilized samples in test tubes and for samples immobilized onto sequencer filters (spotted or electroblotted). It involves relatively little handling, and no thermostated incubator is necessary. The significant advantage is the low yield of internal peptide bond cleavage, also for large polypeptides and proteins, a major difficulty encountered with other pro-

Table 1
Edman degradation of proteins after deacetylation with TFA/methanol

Glucose-6-phosphate dehydrogenase, yeast	Ac-	Ser	Tyr	Asp	Ser	Phe	Gly	Asp	Arg	Val	Thr			
Lyophilized														
Recovery (pmol)		45	40	50	10	55	40	65	10	65	20			
Initial yield (%)		5												
Electroblotted														
Recovery (pmol)		5	10	5	5	10	10	10	+	10	5			
Initial yield (%)		1												
Alcohol dehydrogenase yeast, class III	Ac-	Ser	Ala	Ala	Thr	Val								
Spotted														
Recovery (pmol)		+	235	255	35	220								
Initial yield (%)		10												
Alcohol dehydrogenase, ostrich														
Class I	Ac-	Ser	Thr	Ala	Gly	Lys	Val	Ile	Lys	Cys	Lys	Ala	Ala	Val
Class II[a]	Ac-	Thr		Glu										Ile
Class I lyophilized														
Recovery (pmol)		40	10	45	35	35	30	35	50	25	60			
Initial yield (%)		5												
Mixture of class I and II lyophilized														
Recoveries (pmol)[b]		20	10	30	50	45	50	25	115	50	170	125	125	95
		15		20										65
Initial yields (%)[b]		3												
		2												

(+) Residue identified but no integrator value obtained.
[a]Only residues different in the class I structure are shown.
[b]For class I and II, respectively.

Table 2
Edman degradation after deacetylation with TFA/methanol of peptides corresponding to the N-terminal segments of acetyl-blocked alcohol dehydrogenases

Class III (hagfish)	Ac-	Ser	Lys	Met	Asp	Gly	Gln	Val	Ile	His	Cys	Lys	Ala	Ala	Val
Spotted															
Recovery (pmol)		5	10	10	5	5	5	10	5	5	5	5	5	5	10
Initial yield (%)		3													
Class I (horse)	Ac-	Ser	Thr	Ala	Gly	Lys	Val	Ile	Lys	Cys	Lys				
Lyophilized															
Recovery (pmol)		130	45	190	185	170	180	145	170	+	170				
Initial yield (%)		4													

(-) Residue identified but no integrator value obtained.

tocols [5], which is far more important than the somewhat low initial yield, in allowing interpretation of the amino acid sequences for quite long segments after deblocking.

The N-terminal sequence of a glucose-6-phosphate dehydrogenase [6] was determined at 5% initial yield after deblocking of 1 nmol of the lyophilized protein (Table 1). The same amount without previous deacetylation was tested in the sequencer with no result. The preparation was also analyzed by SDS-polyacrylamide gel electrophoresis and the enzyme was cut from the gel after detection with 1 M KCl and electroblotted onto a Polybrene-treated glass fiber sequencer filter [16]. Edman degradation after deacetylation of the immobilized protein revealed an initial yield of about 1% which is lower than without gel electrophoresis and electroblotting, but still sufficient to make interpretation of the N-terminal sequence for extended segments possible (Table 1). The N-terminal structure of a yeast class III alcohol dehydrogenase [7] was analyzed at 10% initial yield after deacetylation of 2 nmol of the intact protein spotted onto a sequencer filter (Polybrene-treated glass fiber) (Table 1). The blocked nature was verified by five initial cycles of Edman degradation before the filter was removed and the sample in situ deacetylated and reapplied to the sequencer. The method also provides an efficient approach to resolve differences in the amino acid sequence of closely related but blocked isozymes. Thus, class I and II alcohol dehydrogenases isolated from an animal liver [8,9] were successfully deacetylated after preparation separately and in mixture (Table 1). The preparation containing both isozymes was analyzed for 13 cycles revealing isozyme residue differences at positions 1, 3 and 13 (Table 1). An N-terminally acetylated fructose-1,6-bisphosphatase purified from rabbit liver [10] was also tested. After treatment of the enzyme with TFA/methanol, Edman degradation revealed the sequence Ala-Asp-Lys- which shows that the method also works for acetylated N-terminal residues other than Ser and Thr. This finding implies that the mechanism involved is not a simple acid-catalyzed N→O shift of the acetyl group followed by β-elimination which has been suggested for deacetylation of N-terminal Ser and Thr employing neat TFA [5], but rather a more specific reaction involving the methanol.

N-terminally acetylated peptides have also been successfully sequenced after treatment with TFA/methanol (Table 2). Importantly, this is possible when the blocked peptide has been detected by negative results upon initial sequencer degradations. For example, in an HPLC fraction of a tryptic digest of class I alcohol dehydrogenase from hamster, direct sequence analysis revealed no results. The sequencer filter (polyvinylidene difluoride) was then removed from the cartridge and treated with TFA and methanol according to the current protocol. Reapplication of the filter to the sequencer gave the result Ser-Thr-Ala-Gly-Lys, which accounts for the amino acid composition of the peptide. Similarly, the N-terminal sequence of hagfish alcohol dehydrogenase was interpreted for 14 residues (Table 2) at 3% initial yield of an N-terminal peptide generated by GluC digestion of the intact protein [11]. Before that, the sample was analyzed by five cycles of Edman degradation which established its blocked nature, after which the filter (Polybrene-treated glass fiber) was removed and the polypeptide in situ deacetylated followed by reapplication to the sequencer. Another peptide (14 residues) corresponding to the acetylated N-terminal segment of horse liver alcohol dehydrogenase [12] was also deacetylated according to the current protocol, after which sequencer degradation showed an initial yield of 4% (Table 2).

In conclusion, a combination of TFA and methanol provides efficient and specific deblocking of N-terminally acetylated proteins and peptides and allows direct sequence analysis without extensive background even with protein chains of several hundred residues.

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