Qualitative Determination of *N*-Terminal Amino Acids of Peptides and Proteins with Cobalt(III) Chelates

By KEITH W. BENTLEY AND ERNEST H. CREASER

Research School of Biological Sciences, The Australian National University, Canberra City, A.C.T. 2601, Australia

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1. A method of *N*-terminal peptide-bond hydrolysis with the *cis*- β -hydroxyaquo(triethylenetetramine)cobalt(III) ion, i.e. β -[Co(trien)(OH)(OH₂)]²⁺, is reported. The method has been demonstrated with 22 small peptides and ten proteins. 2. The procedure is rapid (an *N*-terminal amino acid determination can be made easily in one day), it involves no acid hydrolysis step and thus no destruction of labile amino acids, and it involves the use of easily prepared inexpensive reagents. 3. The released *N*-terminal amino acids can be identified as their cobalt(III) derivatives, or directly as the amino acid or as their dansylated derivatives. 4. The method is to treat 1 μ mol of peptide or protein with β -[Co(trien)(OH)(OH₂)]²⁺ reagent at pH 8.0, 45°C for 3 h. Addition of 0.5 M-phosphate buffer, pH 10.5 at 45°C for 10 min cleaves the *N*-terminal bidentate amino acid–cobalt complex, which can be identified directly. For greater sensitivity with 10 nmol of peptide the free amino acid is prepared from the complex by treatment (with NaCN (0.1 M, 40°C, 30 min), or H₂S or NaBH₄ (25°C, 5 min), dried, dansylated and the dansyl-amino acid identified by high-voltage electrophoresis. The method is unaffected by the presence of 4–8 M-urea, but will not cleave blocked *N*-terminal acids.

Substitution-labile cobalt(III) ions of the type *cis*-[Co(N)₄(OH)(OH₂)]²⁺, where (N)₄ represents two molecules of ethylenediamine (en), or one of 2,2',2''-triaminotriethylamine (tren), or one of triethylenetetramine (trien), which may be bound either α , i.e. 1,2,3,6 (see Scheme 1), or β , i.e. 1,2,3,4, enhance the hydrolysis of dipeptides, dipeptide esters, amino acid esters and amides at pH 7–8. This results in hydrolysis of the *N*-terminal amino acid, which appears in the final products chelated in the coordination sphere of cobalt(III):

degradation of proteins and peptides. To date, there have only been reports of experiments with model dipeptides at elevated temperatures (55–80°C) and pH 7–8, and no quantitative results have been obtained. Also, because of the temperatures and cobalt concentrations used, a variety of condensation products has invariably resulted. It is the object of our work to explore the potential utility of cobalt chelation in protein degradation and synthesis. The present paper investigates the feasibility of the reactions shown in Scheme 1 for peptide degradation

(Collman & Buckingham, 1963; Buckingham & Collman, 1967; Buckingham *et al.*, 1967; Kimura *et al.*, 1970).

For the *cis*- β -trien system, activation appears to be due to an initial chelation of the terminal α -amino group of the amino acid or peptide moiety followed by displacement by the carboxyl group at the remaining site (Scheme 1). The second reaction is a base-catalysed hydrolysis of the cobalt–amino acid complex from the residual peptide.

The reactions may be accomplished without extremes of pH or temperature and, as they are specific to the *N*-terminal residue, appear to offer great possibilities in the sequential *N*-terminal

and outlines a method of qualitative *N*-terminal identification for peptides and proteins based upon it.

Materials and Methods

Peptide and proteins were of the highest purity obtainable from normal commercial sources. Other chemicals were of A.R. grade. Glycylglycine esters were prepared by the method of Brenner & Huber (1953). Where appropriate the ester was converted into its perchlorate salt by repeated recrystallization from methanol–LiClO₄. The purity of the dipeptide ester salts was confirmed by proton magnetic resonance (p.m.r.) and elemental analysis.

Scheme 1. *N*-Terminal peptide hydrolysis with cobalt(III)tetra-amine chelates

The *cis*- β -diaquo(triethylenetetramine)cobalt(III) perchlorate was originally prepared by the method of Sargeson & Searle (1967) from the *cis*- α -dichloro-(trien)cobalt(III) chloride and the *cis*- α -dinitro-(trien)cobalt(III) chloride. A method has been published by Storm & Ellsworth (1972) and is as follows: to an ice-cold solution of triethylenetetramine (0.5 mol) is slowly added 2 equiv. of 60 % (v/v) HClO₄. After the addition of the HClO₄ is complete, freshly prepared Na₃Co(CO₃)₃·3H₂O (0.5 mol) prepared by the method of Bauer & Drinkard (1960) is added, the mixture stirred for 30 min at 0°C and then warmed to 60°C for 30 min. It is then filtered on a coarse sintered-glass funnel while hot, concentrated to about 200 ml total volume on a rotary evaporator and placed in the cold. After 2 h the red solid is filtered on a coarse, sintered-glass funnel and washed with four 100 ml portions of 95 % (v/v) ethanol.

The red solid is dissolved in 150 ml of hot water, and 45 g of NaClO₄ and 75 ml of methanol are added. It is placed in the cold to recrystallize, then filtered on a coarse, sintered-glass filter, washed with three 50 ml portions of anhydrous methanol and air-dried. At this point the compound, containing only a small amount of NaClO₄ impurity, is of satisfactory purity for use in peptide hydrolysis. One further crystallization from a minimum amount of water gives analytically pure material. When the compound is dissolved in HClO₄ the *cis*- β -diaquo(trien)cobalt(III)³⁺ species is obtained. Adjustment of the pH to

8.0 gives the *cis*- β -hydroxyaquo(trien)cobalt(III)²⁺ species, the active agent in the peptide hydrolysis. The β -[Co(trien)(OH)(OH₂)]²⁺ species is unstable because of dimerization and is prepared just before use. The complexes α -[Co(trien)CO₃]ClO₄, [Co(tren)-CO₃]ClO₄ and [Co(en)₂CO₃]ClO₄ were prepared as described previously (Gillard & Wilkinson, 1963; Kimura *et al.*, 1970; Dwyer *et al.*, 1963).

The reference amino acido(trien)cobalt(III) complexes, with the exception of cysteine, have all been prepared by the procedure described in the text and characterized by the chromatographic and electrophoretic procedures given above, and are fully described elsewhere.

High-voltage electrophoresis was performed on Whatman 3 MM paper in a Pherograph flat-bed, cool-plate refrigerated electrophoresis apparatus, with either formic acid-acetic acid-water (1:3:16, by vol.) or pyridine-acetic acid-water (1:2:100, by vol.) as electrolyte buffers.

For paper chromatography, the cobalt-amino acid complexes were eluted downwards in tanks lined with filter paper soaked with the appropriate eluent to accelerate saturation equilibria. Elution buffers normally used were formic acid-acetic acid-water (1:3:16, by vol.) and butan-1-ol-acetic acid-water-pyridine (50:50:10:1, by vol.).

After elution the spots were detected directly with u.v. light or by spraying with a modified ninhydrin reagent (Troll & Cannan, 1953) and heating at 90°C. After ninhydrin treatment the amino acid complexes

gave a delayed response that required 20–40 min to develop. The proline complex gave no detectable response at the concentrations used.

Spectrophotometric rates were obtained with a Cary 14 or a Cary 16K instrument. Cobalt was measured by using a Techtron AA4 atomic-absorption spectrophotometer. The following radio-meter apparatus was used in the measurement of buffer pH and in pH-stat titrations: TTA₃ electrode assembly, ABU1 autoburette, TTT1 titrator, SBR₂ titrigraph, and PHA scale expander. The glass electrode was bridged with NaNO₃. In the pH-stat titrations the thermostatically controlled vessel (25° ± 0.05°C) was continuously stirred as the titrant (NaOH) was added under an N₂ atmosphere.

Separation of the reaction products was achieved on either a sulphonated polystyrene Bio-Rad analytical Dowex 50W (X2 or X4; 200–400 mesh; H⁺ form) or a carboxymethylated dextran Sephadex CM-25 cation exchanger.

Results

Kinetics

An examination of the efficiency of various *cis*-[Co(N)₄(OH)(OH₂)₂]²⁺ species was done by spectrophotometric measurement of the change in absorbance at 485 nm after the addition of isopropyl glycylglycinate. The results are shown in Table 1. In each case linear plots of log (*D_t* – *D_∞*) against time were obtained over at least three half-lives.

The *cis*-β-hydroxyaquo(triethylenetetramine)-cobalt(III) species condenses with isopropyl glycylglycinate at a rate at least fifty times greater than any of the other species considered. The rate constant for the bis(en) system makes no allowance for the fact that only 60 % *cis*-[Co(en)₂(OH)(OH₂)₂]²⁺ is present at pH 8.0 (Bjerrum & Rasmussen, 1952) and allowance

must be made for this in the comparison. The differences in reaction rates for the consideration of the various [Co(N)₄(OH)(OH₂)₂]²⁺ may be a consequence of the different rates of water exchange {e.g. [Co(en)₂(OH)(OH₂)₂]²⁺ is slower than the [Co(trien)(OH)(OH₂)₂]²⁺ species (Kruse & Taube, 1958; Dwyer, 1971)}.

Analysis of the products of the reaction between the dipeptide ester and the β-[Co(trien)(OH)(OH₂)₂]²⁺ reagent by chromatographic separation and cobalt analysis showed that the expected cobalt-dipeptide ester complex accounted for at least 90 % of the products.

Because of the large rate advantage, the β-[Co(trien)(OH)(OH₂)₂]²⁺ species was used exclusively in subsequent degradative work.

Hydrolysis of the *N*-terminal peptide link by elevated pH gives a rapid recovery of the cobalt-amino acid complex from the residual peptide. The hydrolysis may be effected at pH 8–11. Hydrolysis of the linkage is directly dependent on [OH[–]] (Buckingham *et al.*, 1970) and has been shown to be quantitative at pH 11.0 by cobalt analysis. The cleaved amino acid complexes are all stable at pH 11.0 for 15 h but not at pH values greater than 12 when N–N bonding and ligand displacement occurs.

Amino acid-cobalt(III) complexes may be identified by chromatography or high-voltage electrophoresis (K. W. Bentley & E. H. Creaser, unpublished work) and it was possible to show that the expected amino acid-cobalt complexes were obtained by this procedure from the A chain of insulin, from glucagon and from insulin. However, a more sensitive procedure adopted was to make the free amino acid and then convert it into the dansyl derivative (Gray & Hartley, 1963).

It is possible that an ultra-micro method could be devised with ⁶⁰Co in the complexing reagent but we have not as yet investigated this.

Table 1. Relative rates of reaction between *cis* cobalt complexes (0.2 mM) and isopropyl glycylglycinate (1.0 M) at pH 8.0 (±0.02) and 25.0°C

The reaction to give the cobalt-peptide complex was followed spectrophotometrically at 485 nm, pH 8.0, with an ionic strength of 1.0 with respect to ClO₄[–] and a [Co] of 1 mM. The observed rate of reaction of the α-[Co(trien)(OH)(OH₂)₂]²⁺ was the same as its rate of isomerization to the β-[Co(trien)(OH)(OH₂)₂]²⁺ determined separately, indicating that the β form was the active condensing species and the observed rate of condensation of the α form with isopropyl glycylglycinate was rate-limited by its conversion into the β species. The *cis* to *trans* rearrangement in [Co(en)₂(OH)(OH₂)₂]²⁺ gave a half-life of 340 s at pH 8.0 and an ionic strength of 1.0 (NaClO₄). It was therefore pre-equilibrated for 8 half-lives before initiation of the condensation reaction.

Cobalt complex	<i>t</i> _½ (s)	10 ^{–4} × <i>k</i> _{obs.} (s ^{–1})
β-[Co(trien)(OH)(OH ₂) ₂] ²⁺	34	143.0
α-[Co(trien)(OH)(OH ₂) ₂] ²⁺	1620	4.28
[Co(trien)(OH)(OH ₂) ₂] ²⁺	2310	3.00
[Co(en) ₂ (OH)(OH ₂) ₂] ²⁺	2720	2.55

Removal and identification of the amino acid from the cobalt(III) complex

To give a sensitive means of identification of amino acids, complexes removed during *N*-terminal sequence degradation were investigated for the quantitative release of amino acids from the cobalt(III) complexes. The identification of amino acids in solution is a comparatively well-known and simple operation. Three possibilities were investigated:

(1) Reduction of the cobalt(III) complex to a labile Co^{2+} species by reducing agents such as NaBH_4 , CrCl_2 , VSO_4 , LiAlH_4 , Zn-HCl and lithium, tri-(*t*-butoxy)aluminium hydride. These reductions rapidly release the amino acid. In addition, however, triethylenetetramine and Co^{2+} are liberated, together with the continuous release of bubbles of H_2 gas.

(2) Removal of Co(III) ion from solution by reduction with H_2S or $(\text{NH}_4)_2\text{S}$ releases the amino acid rapidly. However although H_2S is useful for recovering peptides from their Cu(III) complexes (Yamada *et al.*, 1970), the process is messy, releases trien into solution, requires centrifugation or filtration to remove the CoS and $(\text{NH}_4)_2\text{S}$ contaminates the system with an excess of NH_3 . Release of trien involves difficulties when dansylation is used subsequently, as several primary amine compounds than can be dansylated are produced. (However, in practice these compounds migrate much more rapidly than the dansyl amino acids.)

(3) Conversion of the $\beta\text{-[Co(trien)(amino acid)]}^{2+}$ complex into a more stable complex by ligand displacement can be accomplished rapidly by NaCN or HCN gas or more slowly with NaNO_2 or HCl . The NaNO_2 , even at elevated temperatures,

gives a very slow release of amino acids and treatment with HCl (6.0M, 110°C) requires some hours before removal of the amino acid is complete. This is accompanied even under N_2 by substantial loss of tryptophan and loss in part of other amino acids such as methionine and tyrosine.

The HCN generated in the reaction mixture from 1.0M- NaCN and conc. HCl gives a satisfactory removal of amino acids from the cobalt(III) moiety. However, if the use of cyanide is not practical, solid NaBH_4 can be used.

After liberation of the amino acid from the complex, it was identified by formation of the dansyl derivative, which was then characterized by electrophoretic procedures.

Qualitative identification of N-terminal amino acids at the 10 nmol level

To 10nmol of peptide in 0.2ml of 0.2M-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] buffer, pH8, was added 0.2ml of 1.0M- $\beta\text{-[Co(trien)(OH)(OH}_2\text{)]}^{2+}$, pH8.0, and the solution was warmed to 45°C for 3h. Then 0.30ml of 0.5M-sodium phosphate buffer, pH10.5, was added and the solution was left for 40min. Then 0.2ml of 1.0M- NaCN and 0.2ml of conc. HCl were added and the solution was warmed to 40°C for 30min, or 5mg of NaBH_4 was added and allowed to react for 5min. For dansylation the solution was dried *in vacuo* over P_2O_5 and treated in the normal manner.

Results from the procedure are shown in Table 2.

Proteins with blocked *N*-terminal amino acids fail to give a detectable end-group by the cobalt procedure.

Table 2. Peptides from which the *N*-terminal amino acids have been qualitatively removed and identified by cobalt chelation

Cytochrome *c* (horse heart) and ovalbumin were the two peptides from which the *N*-terminal amino acids could not be detected.

(a) Di and tri-peptides

Glycylglycine
Glycyl-L-alanine
Glycyl-DL-leucine
Glycyl-L-phenylalanine
Glycyl-L-histidine
Glycyl-L-tryptophan
L-Methionyl-L-histidine
L-Alanylglycine
L-Phenylalanylglycine
L-Leucyl-L-tyrosine
L-Prolyl-L-hydroxyproline
L-Leucylglycyl-L-phenylalanine
Glycyl-L-leucyl-L-tyrosine
Glycylglycyl-L-arginine
Glycyl-L-tryptophylglycine

(b) Larger peptides

Leu-Trp-Met-Arg-Phe-Ala
Bradykinin
Angiotensin II
Insulin A chain (oxidized)
Glucagon
Insulin (bovine)
Ribonuclease
Trypsin
Pepsin
 α -Chymotrypsin
Peroxidase (horseradish)
Trp-Met-Arg-Phe-Ala
Met-Arg-Phe-Ala

In proteins where there is steric hindrance to attack at the *N*-terminus, the protein may be unfolded by denaturation; urea is normally used for this purpose (cf. Gray, 1967). We have investigated the effects of inclusion of 4–8M-urea in the cobalt *N*-terminal degradation method. Under the experimental conditions used for qualitative hydrolysis neither urea or *NN*-diethylurea form stable identifiable complexes and thus urea can be used in this system to denature the protein under investigation.

Side-chain-modified amino acids such as those found in chemically synthesized proteins form complexes with the cobalt reagent from which the parent amino acid derivative can be liberated by CN^- .

Discussion

The results presented above demonstrate that the cobalt chelate $\beta\text{-[Co(trien)(OH)(OH}_2\text{)]}^{2+}$ is a suitable reagent for detection of the *N*-terminal end-group of a variety of peptides and proteins. By comparison with other methods of *N*-terminal identification its main advantage is that it can be used for acid-labile residues. We have used it to identify *N*-terminal tryptophan and methionine and it is reasonable to hope that it will be equally effective with asparagine and glutamine residues. Further, as complete hydrolysis of the protein does not occur it may be feasible to use the cobalt reagent to shorten a peptide chain before rebuilding it in different ways.

The cobalt reagent was used in the present work under conditions where only the *N*-terminal amino acid is complexed to any significant extent. Preliminary experiments indicate that if the cobalt concentration is raised and the time of incubation is prolonged, other amino acids are sequentially released from the substrate peptide chain. Under these conditions the reagent behaves as a free-running

aminopeptidase. At the present time we do not have methods developed to enable us to use the cobalt reagent as a single-step Edman-type reagent for repetitive degradative steps.

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