The Use of Ninhydrin as a Reagent for the Reversible Modification of Arginine Residues in Proteins

By MARTIN F. CHAPLIN

Department of Biochemistry, University of Ghana, Legon, Ghana

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A simple technique was developed for the specific reversible modification of guanidino groups in proteins involving reaction with ninhydrin. The extent of the reaction is easily determined non-destructively by spectrophotometric analysis. The reagent can also be used for the titration of sterically unhindered thiol groups in proteins.

Development of guanidino-specific reagents is necessary for the determination of the precise role of arginine residues in the stabilization of the tertiary and quaternary structure of proteins and in the allosteric and active sites of enzymes.

The guanidino group of arginine (pK_a 12.5) has been modified by reaction with several diketones (Heinrikson & Kramer, 1974; Yankeelov, 1972). These reagents showed specificity for guanidino groups over amino groups, because the distance between the two carbonyl groups closely matches that of the two unsubstituted nitrogen atoms of the guanidino group. However, the reaction times involved are long (about 24h), and an involved and destructive process is necessary to determine the extent of derivative formation.

The characterization of a colourless adduct between ninhydrin (a triketone) and guanidine has been described previously (MacFadyen, 1950; Ruhemann, 1910). The application of this reaction to the modification of the guanidino group of arginine by ninhydrin has been investigated and the specificity of the reaction determined with amino acids. The reaction has been used to investigate the exposure of the arginine residues in the tertiary structure of trypsin and papain and the effect of the modification on the activity of trypsin.

Materials and Methods

Ninhydrin, benzoylarginine methyl ester and amino acids were from BDH Chemicals, Poole, Dorset, U.K., and were chromatographically homogeneous or AnalaR grade. Trypsin (EC 3.4.21.4) (206 units/mg; 1 unit hydrolyses 1 mol of toluene-4-sulphonyl methyl ester/min at 25°C, pH 8.1, in the presence of 10mM-Ca²⁺) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and papain (EC 3.4.22.2; from papaya latex) from Sigma Chemical Co., St. Louis, MO, U.S.A.

Spectrophotometric absorption measurements were made in a Unicam SP.8000 ultraviolet recording spectrophotometer (Pye Unicam, Cambridge, U.K.).

Tryptic activity was determined by the increase in u.v. absorption at 252 nm of a solution of benzoyl-arginine methyl ester (10 mM) in 50 mM-Tris/HCl buffer, pH 8.1, containing 10 mM-CaCl₂.

The pK_a of ninhydrin was determined by titration of the ninhydrin with NaOH.

A column (30 cm × 1.5 cm diam.) of Sephadex G-25 (coarse grade; Pharmacia, Uppsala, Sweden) was used for gel chromatography, elution being with distilled water (0.5 ml/min at 25°C).

Results and Discussion

Ninhydrin solutions in distilled water gave a u.v. spectrum similar to that obtained by MacFadyen (1950), showing a major peak at 232 nm ($\epsilon = 3.4 \times 10^4$ litre · mol⁻¹ · cm⁻¹), which was invariable with respect to pH, and minor peaks at 254, 294, 308 and 354 nm ($\epsilon = 9.2 \times 10^3$, 7.5×10^2 , 4.8×10^2 and 30 litre · mol⁻¹ · cm⁻¹ respectively, all of which varied slightly with respect to pH. When the ninhydrin solution was allowed to react with solutions of various amino acids or proteins at 37°C, the 232 nm absorption gradually disappeared, and this decrease was used as a measure of the extent of reaction (Table 1).

The reaction between arginine and ninhydrin took place in two stages, an initial rapid reaction to an equilibrium state followed by a very slow reaction (1000-fold slower) of similar velocity (11 μ mol of ninhydrin reacted/min per mol of arginine) to that between glycine and ninhydrin (15 μ mol of ninhydrin reacted/min per mol of glycine) under the same conditions of temperature (37°C) and pH (9.1). During the initial rapid reaction there was no decrease in the 254 nm absorption peak, showing a similarity to the reaction between ninhydrin and guanidine (MacFadyen, 1950), but this peak diminished during the later very slow reaction phase in a

Table 1. *Reaction of ninhydrin with amino acids and proteins*

Ninhydrin (22.4 μM) was allowed to react with an amino acid or protein in a 0.1 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer at 37°C, and the extent of the reaction was determined by the loss in absorbance at 232 nm. n.d., Not determined.

Amino acid or protein	Concn. (mm)	pH	$10^3 \times$ Initial rate of reaction (mmol of ninhydrin reacted/min per mmol of amino acid or protein)	Experimentally determined equilibrium constant
				$(K = \frac{[\text{arginine-ninhydrin complex}]}{[\text{arginine}][\text{ninhydrin}]})$
Glycine	8.90	7.0	<0.003	
Glycine	8.90	9.1	0.015	
Histidine	2.10	9.1	0.025	
Serine	3.15	9.1	0.025	
Lysine	2.28	9.1	0.055	
Papain	0.014	9.1	3.9	
Trypsin	0.0042	9.1	7.4	
Arginine	0.96	4.8	0.0029	n.d.
Arginine	11.5	5.1	0.0043	n.d.
Arginine	11.5	6.4	0.097	n.d.
Arginine	0.192	6.8	0.15	7800
Arginine	0.96	6.9	0.18	n.d.
Arginine	0.142	8.1	1.8	9500
Arginine	0.091	9.1	4.7	10800
Arginine	0.113	9.1	5.9	9500
Arginine	0.176	9.1	4.4	8900
Arginine	0.221	9.1	4.9	6500
Arginine	0.427	9.1	4.4	8100
Arginine	0.615	9.1	4.6	8300
Arginine	0.960	9.1	4.5	9100
Cysteine	0.027	9.1	315	

similar manner to that which occurred in the reaction between ninhydrin and serine, glycine, histidine, lysine and cysteine. This suggested that the initial rapid reaction was due to a specific reaction of ninhydrin with the guanidino group and that the later slow reaction was due to the non-specific reaction of ninhydrin with the free amino groups of these amino acids.

Ninhydrin reacts with arginine very rapidly compared with other amino acids, except cysteine. The initial rate of this reaction was found to be proportional to the concentration of ninhydrin and of arginine and to the OH^- ion concentration, except as the pH approaches 9 (Table 1). The reaction

$\text{p}K_2$ was determined by titration. The independence of K_3 with variation of the pH of the solution allowed $\text{p}K_4$ to be calculated to be about 8.5.

$\text{p}K_3$ could be calculated from the experimentally determined constant (K) involving the equilibrium concentrations of the reagents and product:

$$K = 9 \times 10^3 = \frac{[\text{arginine-ninhydrin complex, total of charged and uncharged species}]}{[\text{arginine, total of all species}][\text{ninhydrin, total of all species}]}$$

rapidly comes to an equilibrium state with an equilibrium constant (K) that was independent of the initial concentrations of the reagents and the pH of the solution (Table 1). These experimentally determined values and relationships are consistent with the equilibria:

K_3 was found to be approx. 10^8 litre/mol, giving $\Delta G^\circ = -20.5 \text{ kJ/mol}$ (-4.9 kcal/mol) for reaction (3).

The rate of the reverse reaction:

Arginine-ninhydrin complex $\rightarrow$ arginine + ninhydrin was deduced to be:

$$k_{-3} \cdot K_4 \frac{[\text{arginine-ninhydrin complex}]}{K_4 + [\text{H}^+]}$$

Table 2. Reaction of ninhydrin with trypsin

Trypsin (2mg) was allowed to react with ninhydrin in a solution (1ml) of 0.1M-NaH₂PO₄/Na₂HPO₄ buffer, pH9.1, or of 50mM-Tris/HCl buffer, pH7.8, containing 10mM-CaCl₂ at 37°C. The excess of ninhydrin was removed by gel chromatography on Sephadex G-25, and the protein fraction was assayed for bound ninhydrin and tryptic activity.

Ninhydrin (mg)	pH	Time of reaction (min)	Activity retained (%)	Ninhydrin bound (mol/mol of trypsin)
12	9.1	30	23	4.0
2	9.1	30	27	2.9
2	9.1	3	77	1.7
10	7.8	3	100	2.4

where k_{-3} was calculated to be 0.04min⁻¹. Although this rate is not slow, at pH8–9, compared with the time for dialysis or gel filtration, it should be noted that excess of reagent will be present until the pH falls.

Trypsin reacted with ninhydrin under various conditions (Table 2) and could be separated from the excess of ninhydrin by dialysis or gel chromatography. The amount of ninhydrin bound to the trypsin was determined from the relative absorbance at 284 and 254nm, with ninhydrin and trypsin being used as standards. The tryptic activity of the complex was determined relative to that of pure trypsin treated similarly, but without ninhydrin (Table 2). It was found that a small extent of reaction (2mol of ninhydrin reacted/mol of trypsin) had little effect on the tryptic activity, but greater extent of reaction resulted in a loss in activity. The initial reaction was equivalent to modification of 1.54mol of guanidino group/mol of trypsin. These results can be compared with the known arginine content of trypsin (2mol/mol; Walsh, 1970) and indicate that these residues are situated externally in the molecule and are not necessary for activity.

The initial reaction between papain and ninhydrin was equivalent to modification of 0.81mol of guanidino group/mol of papain and no free thiol. This

agrees with Heinrikson (1971) in that the free thiol group (Arnon, 1970) of papain is buried deep in the molecule, but the rate indicates that most of the arginine residues (12mol/mol; Arnon, 1970) are also sterically hindered at least as far as reaction with ninhydrin is concerned.

The reaction between cysteine thiol groups and ninhydrin is rapid and goes essentially to completion. It can be used for the modification and titration of these groups, when they are not sterically protected or for the determination of the degree of steric protection.

The ninhydrin–arginine reaction can be used for the titration of sterically unprotected arginine groups in proteins and for the modification of proteins for investigation into their structure/activity locus. After reaction at alkaline pH, the protein can be stored for some time at acid pH for study free from excess of reagents, as the reverse reaction is slow at low pH (less than 1% per day at pH4.5) and the unmodified protein can then be re-formed by dialysis at basic pH. This reversible reaction can usefully be applied to kinetic studies on the allosteric and active sites of enzymes, with low interference due to side reactions. These applications, involving a relatively cheap and readily available reagent, are simplified by the ease with which the rate and degree of reaction can be determined non-destructively by u.v. spectrophotometry.

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