FORMATION OF NON-AMIDINE PRODUCTS IN THE REACTION OF PRIMARY AMINES WITH IMIDO ESTERS

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SUMMARY: Imido esters are widely employed for the chemical modification of amino groups in proteins between pH 7-10. We have found that near pH 8 the initial products of reaction of simple primary amines with imido esters are N-alkyl imidates which subsequently react either with ammonia to yield the expected amidine or with water to form free amine. In contrast, near pH 10 amidine formation occurs more rapidly and in better yield, apparently without the accumulation of an intermediate. The observed mechanism of amidine formation implies the possible occurrence of novel side reactions and suggests improved conditions for protein amidination.

Treatment of proteins with imido esters constitutes an important and extensively employed method of chemical modification. The reaction is reported (1-3) to result in a very specific and nearly quantitative conversion of amino groups to amidine functions at pH values near neutrality.

Conditions specified in the literature for quantitative amidination of proteins vary widely but typically involve reaction times of less than one hour (4) to several days (5) at pH values between 7 and 10. We observed, however, that alkyl acetimidates react very rapidly with water in this pH range. It was therefore not clear how reactions of proteins with imido esters could apparently occur over a period of many hours. We therefore felt that a re-examination of the mechanism of protein amidination was in order. In this communication, we report our studies of the reac-

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tions of imido esters with simple primary amines under conditions similar to those used in protein modifications. The accompanying communication presents the results of similar investigations of the mechanism of amidination of a protein.

MATERIALS AND METHODS

Imido esters were synthesized by the Pinner method (6) or were purchased from Eastman. Proton nmr spectra were obtained using either a JEOL MH-100 or a Varian T-60 nmr spectrometer.

RESULTS

Stability of Acetimidates in Aqueous Solution. The disappearance of ethyl acetimidate in aqueous solution was monitored by proton nmr as a function of pH at 22° and 35° (Figure 1). The half-time for disappearance of ethyl acetimidate was 2-5 minutes below pH 8.5 and increased substantially at higher pH values. The major

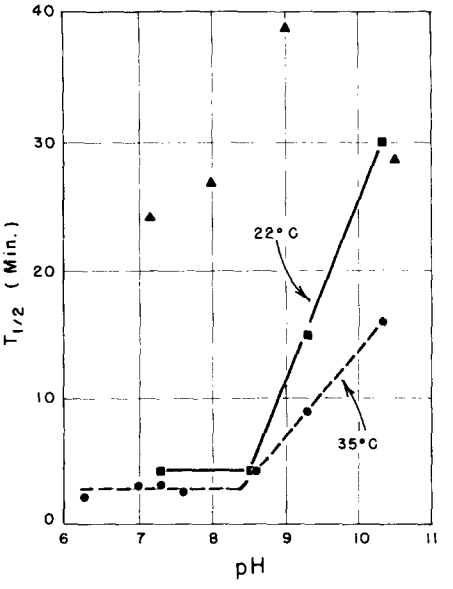

Figure 1. Half-times for disappearance of ethyl acetimidate (initially 0.2 M) in buffered aqueous solution at 22° or 35° as determined by proton nmr. The buffers employed were 0.5 M NaPi below pH 9 and 0.5 M Na_2CO_3 above pH 9. Identical half-times below pH 9 were obtained using N-ethylmorpholine·HCl buffers. Literature (1) values for methyl acetimidate hydrolysis at 25° are indicated by triangles.

products of the reaction were ethyl acetate and acetamidine (presumably formed by reaction of ammonia with ethyl acetimidate).

Reaction of Acetimidates with Primary Amines. The reactions of methyl or ethyl acetimidate with an equivalent amount of n-propylamine or ε-caproic acid at initial pH values of 8 (Table 1) or 10 were monitored by proton nmr. At the lower pH value, the acetimidates reacted very rapidly (85% complete in less than one

TABLE 1

Apparent Half-Times for Reactions of Alkyl Acetimidates Occurring in Solutions of Primary Amines at pH 8 †

Reaction	Alkyl Group	Approximate Half-Time of Reaction, Minutes: n-Propylamine	ε-Aminocaproate
Acetimidate with Amine	ethyl	<2	<1.2
	methyl	<1.2	<1
Acetimidate "Hydrolysis"	ethyl	4.5	3
	methyl	2	1.3
N-Alkyl Acetimidate Disappearance	ethyl	7	7.5
	methyl	5	4
N-Alkyl Acetamidine Formation	ethyl	4.5*	3.5*
	methyl	1.5*	1**
Free Amine Formation	ethyl	8.5	5
	methyl	3	(not det'd)

† All experiments were performed in 2 M NaPi, initial pH 8.2, final pH 7.4 ± 0.1, at 22° initially, then in nmr probe at 35°. The initial concentration of amine was 0.38 M in all cases; the initial concentration of imidate was either 0.40 M (for ethyl) or 0.46 M (for methyl). In each case, an amount of the acetimidate essentially equivalent to the total amount of amine was consumed in N-alkyl imidate formation within 2-3 minutes of mixing. In all cases, about 70% of the N-alkyl imidate reacted to give free amine, and about 30% gave N-alkyl acetamidine. Not all reactions showed first order kinetics.

* After 2 minute lag

** After 1 minute lag

minute) and essentially quantitatively to form N-alkyl imidates derived from the primary amines. The N-alkyl acetimidates then underwent slower reactions (half-time about 7 minutes for disappearance of ethyl imidates, about 4 minutes for methyl imidates) to give approximately two parts free amine and one part N-alkyl acetamidine. In all cases, there was an initial lag of about 1-2 minutes before N-alkyl acetamidine was produced in significant amounts.

In contrast, reaction of ethyl acetimidate with one equivalent of ε-aminocaproic acid at initial pH 10 (0.5 M borate buffer) gave a two- to three-fold higher yield of N-alkyl acetamidine (60% vs. 20-30%) than reaction at pH 8. The formation of N-alkyl acetamidine was more than 90% complete within three minutes of mixing.

DISCUSSION

Our proton nmr studies of the reactions occurring in buffered aqueous solutions of imido esters have shown that, under conditions of use in protein chemistry, alkyl acetimidates decompose in aqueous solution as much as an order of magnitude faster than indicated in the literature (1).

The investigations of the reactions occurring in buffered aqueous solutions containing approximately equivalent amounts of imido ester and primary amine indicate that acetamidines are formed more rapidly and in higher yield at pH 10 than at pH 8. This difference indicates a change in the effective mechanism of the amidination reaction, in agreement with the work of Hand and Jencks (7) on benzimidates. At the lower pH value, formation of amidine clearly proceeds via an intermediate N-alkyl imidate (Figure 2, upper right). At the higher pH value, little (<5%) N-alkyl imidate was detected by proton nmr, and it is likely that the amidine

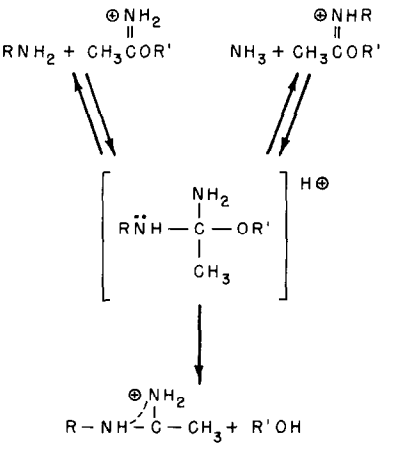

Figure 2. Proposed mechanism for the reaction of primary amines with imido esters.

product is formed directly (Figure 2). At the intermediate pH values (pH 8-9) often used in protein modification, a mixed mechanism will operate.

The reactions between n-propylamine or ε-caproic acid and acetimidates should constitute valid models for the reaction of amino groups in enzymes, e.g., the ε-amino groups of lysine residues, with imido esters since lysine ε-amino groups in proteins generally occur at the surfaces of the molecules with the side-chains pointing into solution. Preliminary communications with other workers suggest that the protein amidination mechanism we have determined for alkyl acetimidates also applies to other imido esters, including ones used in cross-linking and membrane studies. It must therefore be inferred that treatment of proteins with imido esters at pH values near neutrality will initially result in the conversion of many protein amino groups into N-alkyl imidates rather than amidines.

IMPLICATIONS

Our findings and conclusions have important implications in

the design and interpretation of experiments involving chemical modification of proteins using imido esters. The prolonged reaction times often used for protein amidinations near pH 10 appear unnecessary and undesirable. More importantly, it is clear that, no matter how large an excess of imido ester reagent is used, quantitative amidine formation is impossible using a single addition of reagent near or below pH 8. The availability of protein amino groups cannot be directly quantitated by the extent of amidine formation under these conditions, as has been suggested (8). If rapid, quantitative acetamidine formation is desired, the reaction should be carried out at pH 10. Failing that, a pH value as high as possible for the protein under study should be used, and/or multiple additions of reagent at appropriate intervals.

Unexpected side reactions very likely accompany amidine formation when the reaction is carried out near pH 8. Crosslinking of protein molecules could occur with monofunctional imido esters as the result of reaction of a protein amino group with a nearby protein N-alkyl imidate, especially if a low ratio of imido ester to amine is employed (see reference 9 for a case in which such crosslinking may have occurred). Similarly, covalent over-incorporation of a labeled imido ester could occur through multiple modification of a single lysine ε-amino group by reaction of the initially formed N-alkyl imidate with acetimidate (cf. reference 10). After one or several such steps, reaction with ammonia would give a conjugated "polyamidine" system.

Alkyl diimido esters used as cross-linking reagents can be expected to react by the same mechanism as alkyl acetimidates. An important consequence of this fact will be inefficient cross-linking near pH 8. Conversion of some amino groups to diamidines can also be expected, and will result in formation of macromolecules with a range of charges.

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