FORMATION OF NON-AMIDINE PRODUCTS IN THE CHEMICAL MODIFICATION OF HORSE LIVER ALCOHOL DEHYDROGENASE WITH IMIDO ESTERS

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SUMMARY: The reaction of imido esters with horse liver alcohol dehydrogenase (LADH) and other proteins is widely considered to involve direct conversion of amino groups to amidine functions. We have shown that the 14-fold activated form of LADH which is produced when the modification is carried out near pH 8 contains primarily N-alkyl imidate, rather than amidine, moieties. Fully acetamidinated LADH, which is formed directly at pH 10, or by multiple modification at pH 8, is 6-fold activated. The observed mechanism of amidine formation suggests a re-evaluation of various conclusions drawn from studies of protein amidination.

In the accompanying communication, we reported that near pH 8 the initial product of reaction of an acetimido ester with a simple primary amine is an N-alkyl imidate. In view of this result, we felt it likely that reactions of the lysyl ε-amino groups of proteins with imido esters near pH 8 also yield non-amidine products. We report here our studies of the reaction of horse liver alcohol dehydrogenase (LADH) with acetimido esters, in which we have demonstrated that near pH 8 the initial product of the reaction is an enzyme containing primarily N-alkyl imidate rather than amidine functions.

MATERIALS AND METHODS

Horse liver alcohol dehydrogenase (specific activity approximately 2.6 U/mg) was purchased from Boehringer Mannheim Corporation and assayed by a procedure identical to that of Plapp (1). Imido esters were synthesized by the Pinner method (2) or were purchased from Eastman Organic Chemicals. Pronase CB was obtained

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from Calbiochem and aminopeptidase M from Rohm and Hass. Total enzymatic protein hydrolyses were performed in 0.5 M N-ethylmorpholine·HCl at pH 8.2 using Pronase and aminopeptidase M. Amino acid analyses were run on a Beckman Model 120C instrument.

RESULTS

In accord with the results of Plapp (1, 3-4), we found that treatment of LADH with a large excess of ethyl acetimidate at pH 8 led to activation of the enzyme which reached a maximum (approximately 14-fold) after about 15 minutes. The activity then decreased over a period of several hours to give a preparation with about 3-fold activation which did not lose appreciable further activity on prolonged standing (Figure 1). In contrast, amidination at pH 10 resulted in an activation (about 6-fold) which was complete in about 5 minutes. The activity of this second preparation did not decrease on standing (Figure 1).

A stable preparation of LADH activated about 6-fold could be prepared at pH 8 by making multiple additions of the imido ester. Until a stable preparation had been achieved, each addition resulted in a transient activation (Figure 2). In contrast, multiple additions of reagent to the stable preparation of LADH formed at pH 10 had no effect on the activity of the preparation.

The 6-fold activated preparations of LADH prepared either by a single treatment with ethyl acetimidate at pH 10 or by multiple treatments at pH 8 showed no loss of activity when stored for several months at 5° C in the presence of NADH and isobutyramide at pH 7. Samples of these preparations were subjected to total enzymatic digestion. Amino acid analysis showed 28 ε-acetamidinated lysines and 2 free lysines per polypeptide chain. Samples of partially acetamidinated LADH prepared at pH 8 by one or several treatments with ethyl acetimidate were also analyzed after they had been allowed to stand long enough so that their activity had stabilized (net activation of less than six-fold). There was a

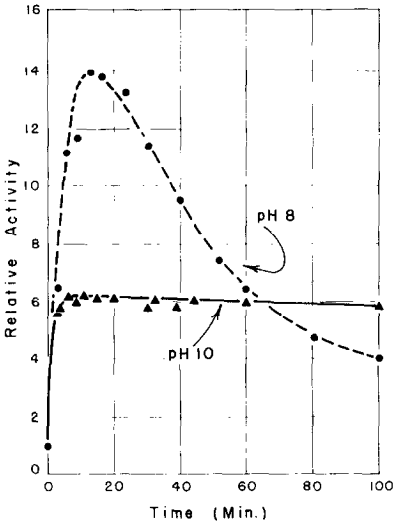

Figure 1. Reaction of ethyl acetimidate (250-fold excess over amine) with LADH (1 mg/ml) in 0.5 M NaPi, pH 8, or 0.5 M Na_2CO_3, pH 10. A transient high initial activation was observed for reaction at pH 8 in 0.5 M N-ethylmorpholine·HCl which was qualitatively similar to that shown for phosphate buffer at the same pH.

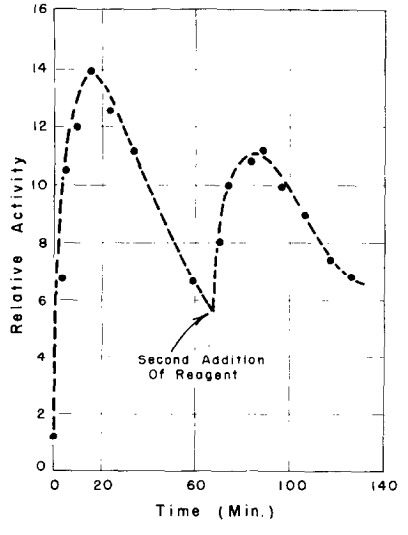

Figure 2. Reaction of ethyl acetimidate (0.1 M) with LADH (1 mg/ml) at pH 8 in 0.5 M NaPi. Addition of ethyl acetimidate (0.1 M) was repeated after 67.5 minutes.

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direct correspondence between the extent of stable activation and the number of ε-acetamidinated lysines formed.

As had been previously demonstrated by Plapp (1, 3-4), we found that LADH acetamidinated in the presence of excess NADH and isobutyramide is slightly deactivated. For example, a preparation of LADH treated with seven portions of ethyl acetimidate at pH 8 had a specific activity of 1.2 U/mg. Removal of NADH and isobutyramide from this selectively modified enzyme gave a preparation which could be activated by ethyl acetimidate at either pH 8 (about 25-fold maximum activation) or pH 10 (about 9-fold activation). Plots of relative activity versus reaction time were qualitatively very similar to those of Figure 1. A single lysine residue per subunit is modified in this second step (Browne and Kent, in preparation; also cf. reference 5).

DISCUSSION

Our experiments on chemical modification of LADH using ethyl acetimidate show clearly that products other than amidines are formed initially under conditions commonly used to achieve overall amidination of proteins.

Fully acetamidinated LADH, in which 28 of the 30 lysine ε-amino groups per subunit have been modified*, has six times the activity of the native enzyme and is of comparable stability. It is produced rapidly when LADH is treated with ethyl acetimidate at pH 10. In contrast, the species initially formed when LADH is treated with ethyl acetimidate at pH 8 is approximately 14-fold activated and decomposes relatively rapidly to yield a preparation of partially amidinated LADH, which can be further activated by subsequent additions of the imido ester.

The changes in activity which accompany modification of LADH with imido esters apparently reflect primarily reactions occurring

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* The α-amino groups of the identical polypeptide chains are acetylated in the native enzyme.

at a single lysine residue per subunit. Thus, changes in activity which accompany ethyl acetimidate treatment of selectively acetamidinated LADH, in which all but one of the available lysines have previously been acetamidinated, are qualitatively very similar to those which occur when ethyl acetimidate is used to modify native LADH.

A coherent explanation of our investigations of LADH amidination which is also consistent with our studies of reactions of simple amines with imido esters (accompanying communication) is that the initial reaction products when proteins are treated with imido esters at pH values near 8 are protein N-alkyl imidates rather than amidines (Figure 3). The protein N-alkyl imidate then partitions approximately equally between reaction with ammonia to form amidine and hydrolysis to generate free amine. A partially amidinated protein results. The correspondence between the extent of stable activation and the fraction of available lysines converted to the ε-acetamidines in partially modified LADH shows that the protectable lysine responsible for the activity changes reacts

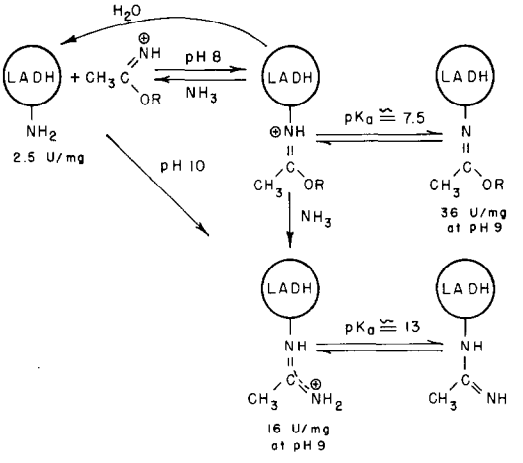

Figure 3. Proposed mechanism for the reaction of LADH with imido esters.

in the same way as the 27 other available lysines. All or nearly all of the 28 available lysines per subunit therefore react according to the proposed mechanism. At higher pH values (near pH 10) protein N-alkyl imidates are not formed to an appreciable extent, and amidination proceeds rapidly and essentially quantitatively.

Our results in this and the accompanying communication cast doubt on various conclusions drawn from studies of protein amidination. For example, some of the interpretations of experiments in which the "active site" lysine of LADH had been modified using imido esters (1, 3-4) are questionable if enzyme N-alkyl imidates, rather than amidines, were being studied. In view of our results, this possibility must be considered. The biphasic kinetics observed for modification of lysines in fibrinogen with ethyl acetimidate (6) could reflect a mixed reaction mechanism rather than different populations of lysines. Similarly, the pH-rate profiles reported (7) for inactivation of isocitrate dehydrogenase with imido esters are those expected for primary amines of normal pK_a (ca. 10.5). They do not suggest that the reacting amino group have an abnormally low pK_a of about 8, as was claimed (7).

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