ARYL α-HALOALKYL KETOXIMES AS ENZYME MODIFYING AGENTS. THE REACTION OF α-HALOACETOPHENONE OXIMES WITH THE ACTIVE SITE OF PAPAIN

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Summary: Syn***-α-chloroacetophenone oxime has been found to inactivate papain rapidly at pH 7 and 25.0° in a 1:1 stoichiometric fashion as measured by rate assays with p-nitrophenyl N-benzyloxycarbonylglycinate and sulfhydryl group titrations with 5,5'-dithiobis-(2-nitrobenzoic acid). By the use of a ^{14}C label in the halomethyl function a similar stoichiometric reaction with papain could be demonstrated for syn-α-bromoacetophenone oxime despite the rapidity of the competitive nonenzymatic solvolysis of the latter compound under the conditions employed. These results indicate that a new class of reactive modifying agents, α-haloalkyl oximes, can be used for the selective alkylation of catalytically essential functional groups in enzyme active sites.

Despite the frequency with which α-haloalkyl carbonyl compounds have been employed for the alkylation of reactive functional groups in enzyme active sites (1), the potential use of closely related derivatives, the α-haloalkyl oximes, for active site modification has not received attention. In the present article we wish to present results which show that the representative aryl α-haloalkyl ketoximes, α-bromo- and α-chloroacetophenone oxime, react with the active site of papain in a 1:1 manner with concomitant inactivation of the enzyme.

The results obtained for the inhibition of papain by reaction with added α-

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***In this article syn refers to the isomer having the alkyl group cis to the oxime oxygen.

TABLE 1

INHIBITION OF PAPAIN BY SYN-CHLOROACETOPHENONE OXIME IN 0.067 M PHOSPHATE BUFFER, pH = 7.0, 10^{-4} M, 25.0°[a]

10^5 [oxime] M	% Original Enzymatic Activity Remaining		% Original Sulfhydryl Content Remaining	
	Found[b]	Expected[c]	Found[d]	Expected[c]
0.00	100	100	100	100
1.78	47	45	43	45
7.13	42	0	0.5	0
1.78[e]	100	45	100	45

[a] $[papain]_o = 3.28 \times 10^{-5}$ M, 2.42% CH_3CN. Purification method: (Blumberg, S., Schechter, I. and Berger, A., Eur. J. Biochem., 15, 97 (1970)).

[b] The activity was measured by assay with p-nitrophenyl N-benzyloxycarbonylglycinate.

[c] Expected values are based on a one to one reaction of the oxime with the active site sulfhydryl group of the enzyme.

[d] These values were determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid). (Ellman, G. L., Arch Biochem. Biophys., 82, 70 (1959)).

[e] This sample of oxime was preincubated at pH 7.0 for 150 sec before addition to the enzyme solution.

chloroacetophenone oxime at pH 7.0 are summarized in Table 1. In view of the marked lability of this oxime to nonenzymatic solvolysis of the halomethyl function (2, 3) the observation of the effectiveness of the inactivation process is indeed remarkable. The solvolytic reactivity of α-chloroacetophenone oxime and the unreactivity of its solvolysis products are illustrated well by the finding that preincubation of the oxime at pH 7.0 for 150 sec before addition to the enzyme solution prevented the inhibition of the enzyme. The parallelism seen between the loss of

enzymatic activity and of sulfhydryl group content strongly suggests that the catalytically essential group which was alkylated was the active site Cys 25 residue (4,5).

When syn-α-bromoacetophenone oxime was reacted with papain, it was found, not surprisingly, that competitive nonenzymatic solvolysis played a more significant role than was the case for the chloro derivative. For example, addition at 25.0° of 2.98×10^{-5} M syn-α-bromoacetophenone oxime to 2.64×10^{-5} M papain in 0.067 M phosphate buffer, pH 7.0, containing 9% CH_3CN, resulted in 83% inhibition of the enzymatic activity as measured by assay with p-nitrophenyl N-benzyloxycarbonyl-glycinate (6). To establish firmly the stoichiometry, therefore, of the inactivation reaction the modification of the enzyme with the ^{14}C-labelled α-bromo oxime was undertaken.

The synthesis of α-bromoacetophenone labelled with ^{14}C in the bromomethyl group was carried out starting with [1-^{14}C] acetyl chloride (0.25 mCi, 3.4 mg) purchased from New England Nuclear, which after dilution with 730 μl of unlabelled acetyl chloride was condensed at 45-50° with benzene (20 ml) with catalysis by $AlCl_3$ (7 g). Treatment of the resultant labelled acetophenone in CCl_4 (15 ml) with bromine (15 g) using $AlCl_3$ (12 mg) as a catalyst, followed by reaction of the α-bromoacetophenone produced with hydroxylamine sulfate (3.4 g) in methanol (30 ml) gave ^{14}C-labelled α-bromoacetophenone oxime (338 mg) with mp 98.5-99° (lit [7] mp 97-98°). The specific activity of the oxime obtained was 100 cpm/μg using Bray's solution as the liquid scintillator [8].

In typical experiments 10 μl of an CH_3CN solution of ^{14}C-labelled α-bromoacetophenone oxime was added to 1 ml of 1.45×10^{-4} M papain in 0.067 M phosphate buffer, pH 7.0, containing 10^{-4} M EDTA. The oxime concentration ranged from 9.18×10^{-5} M to 3.66×10^{-4} M. The solutions resulting from the reaction of the oxime with papain were gel-filtered on a Sephadex G-25 column. Measurements

of the enzymatic activity of the gel-filtered solutions using p-nitrophenyl N-benzyloxycarbonylglycinate as the assay substrate in combination with the results of liquid scintillation counting of these solutions showed that the inactivation of the enzyme corresponded to the incorporation of 0.98 ± 0.23 oxime group per molecule of the enzyme. In other words, as in the case of modification with α-chloroacetophenone oxime, inactivation of papain using the α-bromo species occurred with essentially 1:1 stoichiometry at pH 7.0.

As illustrated by equation 1 where X is halogen and Nu is a nucleophile, the reactions of α-haloacetophenone oximes with several oxygen, nitrogen, and sulfur nucleophiles in aqueous solution have been shown to proceed by way of the formation of an intermediate, suggested to be α-nitrosostyrene, which reacts more rapidly in the s-trans conformation than in the s-cis, giving the thermally unstable anti-alkyl aryl ketoxime as the product (3,9). Under the conditions studied the rate-controlling step has been the formation of the α-nitrosostyrene intermediate. It is conceivable that the inactivation of papain by the α-haloacetophenone oximes occurs by a route like that of equation 1 with highly effective trapping of the α-nitrosostyrene intermediate by the active site sulfhydryl function. An argument against the interpretation of the inactivation of papain by α-haloacetophenone oximes in terms of the route of equation 1 is that since a common intermediate, α-nitrosostyrene, would be invoked for the reactions of both syn-α-bromo and syn-α-chloroacetophenone oxime, it is hard to see why nonenzymatic solvolysis of the α-bromo compound would compete more effectively with the enzymatic process than is the case for the α-chloro species. An alternative mechanistic possibility is that the direct displacement of halide from the α-haloacetophenone oximes by papain's active site sulfhydryl group with concomitant alkylation of the latter function occurs without the intermediacy of α-nitrosostyrene. In this event, the oxime hydroxyl function might remain syn to the alkyl group in the modified enzyme.

Kinetic experiments to distinguish between the alternative mechanistic hypothesis for the inactivation of papain by aryl α-haloalkyl ketoximes are in progress in our laboratory.

$$\text{Ph-}\overset{\overset{\text{N-OH}}{\|}}{\text{C}}\text{-CH}_2\text{X} \xrightarrow{\text{-HX}} [\text{Ph-}\overset{\overset{\text{NO}}{|}}{\text{C}}\text{=CH}_2] \xrightarrow{\text{Nu}} \text{Ph-}\overset{\overset{\text{HO-N}}{\|}}{\text{C}}\text{-CH}_2\text{Nu} \qquad (1)$$

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