EVIDENCE FOR THE HIS-57 SIDECHAIN ACTING AS A GENERAL BASE IN THE "AGEING" AND REACTIVATION REACTIONS OF CARBONATE-INHIBITED α-CHYMOTRYPSIN

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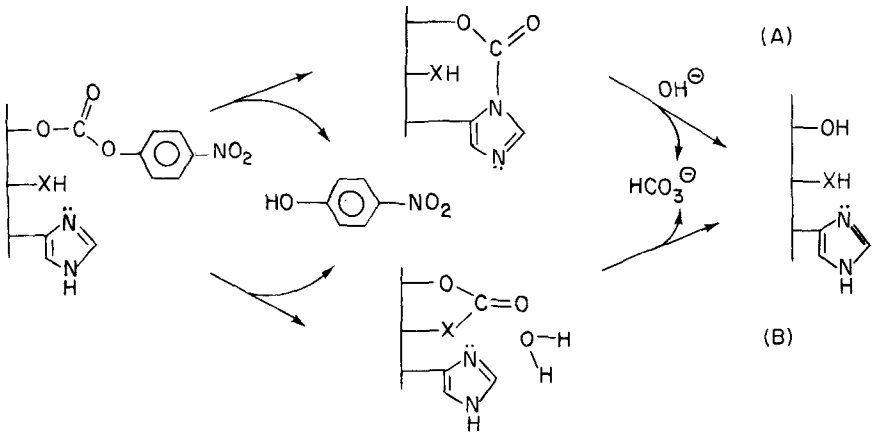
SUMMARY:
The chymotrypsin-catalyzed "ageing" reactions which follow inhibition with phosphate or carbonate esters involve an active site group of pK_a near 7, implicating the imidazole of His 57, as with deacylations. With bis(p-nitrophenyl) carbonate recent evidence is in conflict over whether in the "ageing" reaction the imidazole acts as a general base or as a nucleophile. A crucial in situ reactivation experiment has been carried out at high pH to distinguish between these cases. An ionizable enzyme group of pK 7.4 is involved in the reactivation process for at least 80% of the enzyme. This indicates that imidazole acts mainly as a general base in the ageing and reactivation reactions of labile carbonate di-esters with chymotrypsin.

Labile phosphate esters, e.g., DFP, react readily and specifically with the active sites of serine esterases to give inactive alkyl or acyl phosphate-enzyme derivatives. The "ageing" reaction which follows phosphorylation produces an enzyme no longer reactivatable by strong nucleophiles. The mechanism of this ageing reaction has been studied in considerable detail in recent years (1-4). Labile carbonate esters such as bis(p-nitrophenyl) carbonate, like their phosphate analogs, also undergo kinetically separable acylation and "ageing" reactions, the latter being the release of the second mole of alcohol product, producing an inactive enzyme. In ageing reactions with carbonate esters the inactive enzyme then spontaneously reactivates in a first-order process (4). The identity of the groups involved is quite uncertain, but Fife, Hutchins, and McMahon (5) have argued for direct attack of the His 57 in the ageing reaction to produce an acyl-imidazole intermediate. The reactivation then must necessarily involve spontaneous (non-enzymically catalyzed) attack of water or hydroxide. On the other hand, the data of Wedler and Bender (6) have been interpreted as

consistent with a general base role for the imidazole. Thus imidazole is free to catalyze the reactivation, presumably as a general base again. These two different mechanisms are presented as:



Obviously, the pH-rate profiles for the reactivation reactions in the two cases will be rather different. The plot of log k vs pH for the pathway involving the acyl-imidazole intermediate (A) will be a straight line of slope = 1.0, but for the enzymic general base mechanism (B) it will be sigmoidal with a plateau at high pH. To resolve this conflict, crucial experiments were carried out at high pH to clearly distinguish which pH-rate profile obtains, in fact.

MATERIALS AND METHODS:

Alpha-chymotrypsin was a Worthington product, 3x crystallized, salt free. Bis(p-nitrophenyl) carbonate was synthesized from phosgene and sodium p-nitrophenoxide (6). Methyl N-acetyl-L-phenylalaninate was a gift of Miss Laura Uretsky. Other reagents were of the highest purity.

The kinetics of reactivation were observed on a Radiometer pH stat, fitted with a 0.25 ml autoburette unit, the reaction vessel being thermostatted at 25 ± 0.1°C. The substrate (N-Ac-L-Phe ME) was dissolved directly into degassed aqueous 0.2 M KCl, two ml of which was used in each reaction run. The final concentration was usually about 5-10 mM. The carbonate-inhibited enzyme, $E_o = 10^{-4}$ M, was prepared at pH 5.0 with a 10% excess of bis(p-nitrophenyl) carbonate. An aliquot of this solution was taken immediately and transferred to the specific substrate assay vessel on the pH stat. Enzyme was invariably inhibited 80% or more at the earliest point observable in the turnover assay. The amount of active enzyme at a given time was calculated from the slope of the turnover reaction, corrected for spontaneous hydrolysis of substrate. The rate of reactivation was thus the rate of approach of the slope to that for fully active enzyme. This was a first-order process to greater than four half-lives.

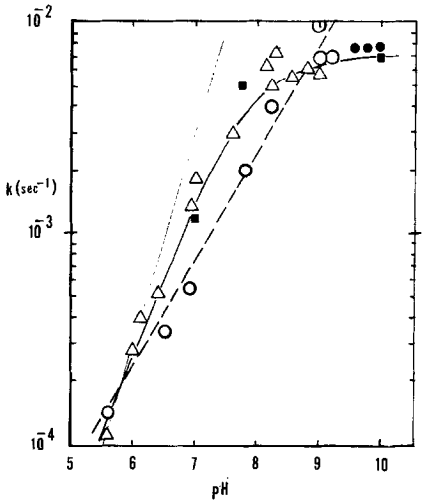


Figure 1:

The rates of reactivation from bis(p-nitrophenyl) carbonate-inhibited chymoytrypsin at various pH values. The data of Fife et al. (5) and Wedler and Bender (6) are replotted as open circles and open triangles, respectively. The reactivation rates observed by in situ assay (see text) are the closed circles. Rate of change of A_{245} (our data) are plotted as closed squares.

RESULTS:

A comparison of the log k vs pH data for the reappearance of active enzyme as determined by various means is presented in Figure 1. The phenomena observed by Fife et al. (ref. 5, Figure 3), by proflavin binding and absorbance changes at 245 nm are plotted as open circles and those observed by Wedler and Bender (ref. 6, Figure 6) by in situ assays, proflavin binding, and carbonate ester turnover reactions are plotted as open triangles. Surprisingly, on this replot, the data interpreted as hydroxide-catalyzed reactivation (5) show a slope (broken Line) of 0.5, not the slope of 1.0 expected for such a process. The data of Wedler and Bender are more suggestive of a sigmoidal curve (solid line), but not absolutely conclusive proof of this. Overall, the agreement between data points from different laboratories is at worst within a factor of 2. However, data at higher pH values were needed for a clear interpretation of the mechanism. Additional data above pH 9.2 were clearly needed, for one to be able to distinguish between the hydroxide-catalyzed and imidazole-catalyzed processes.

The in situ reactivation assays described in Materials and Methods were carried out at pH 9.6, 9.8, and 10.0. The rates of reactivation of carbonate inhibited enzyme at all these pH values was a first-order process with essentially identical rate constants, $k = 7.5 \pm 0.8 \times 10^{-3}$ sec^{-1}. These data

are plotted in Figure 1 as closed circles. Our observations of the rate of change of absorbance at 245 nm at pH 7.0, 7.8, and 10.0 are also plotted in Figure 1 as closed squares. A plateau in the rate of reactivation at high pH is clearly indicated, not a dependence on hydroxide concentration. These data argue very strongly for the His 57 sidechain acting as a general base in both the ageing and reactivation steps for the carbonate-chymotrypsin reaction.

DISCUSSION:

In summary, the evidence which points to a general base role for the imidazole of His 57 is: (a) The presence of added methanol as a nucleophilic analog of water (ref. 6) accelerates ageing by 7-fold and reactivation by 15-fold -- methanol should have no effect on the ageing rate if imidazole acts as a nucleophile. (b) The ageing reaction shows a kinetic isotope effect in D_2O of 2.3.* (c) The pH-rate dependence of reactivation is clearly a sigmoidal one, with pK_a 7.4, indicating a free active site imidazole. (d) No indication of acid-catalyzed reactivation is apparent at pH 5 or below, which might be expected due to protonation of the acyl-imidazole if it occurred.

Those phenomena which are not well understood at present are: (a) Exactly what is causing the absorbance changes at 245 nm. -- this may be due to protein conformational changes or other effects which occur upon acylation or ageing. It appears that the general base pathway is the predominent but not exclusive one in this reaction. The enzyme may thus partition itself between two pathways, the nucleophilic one being a rather minor one, but sufficient to allow some observable acyl-imidazole intermediate to form. (b) The identity of the enzymic group X apparently involved in the aged enzyme species.

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* This observation is consistent with but does not constitute proof for a general base mechanism.