β-LACTONES AS REAGENTS IN PROTEIN CHEMISTRY. THE INACTIVATION OF PEPSIN WITH β-PROPIOLACTONE

Richard N. Armstrong and Emil Thomas Kaiser*

Departments of Biochemistry
and Chemistry
University of Chicago
Chicago, Illinois 60637

Treatment of pepsin with β-propiolactone results in the alkylation of 1-2 methionine residues and the esterification of an aspartate residue. The modified enzyme is characterized by a marked (20-fold) decrease in catalytic efficiency toward benzyloxycarbonyl-histidyl-p-nitrophenyl-alanyl-phenylalanyl methyl ester. The results suggest that the aspartate alkylated is in the active site.

The unusual reactivity of β-propiolactone (oxetan-2-one) has recently led to its use as a methionine specific protein modifying reagent (1). However, its limited use has left the potential of β-lactones as enzyme probes largely unexplored. Reaction of the oxetane ring with nucleophiles can occur with fission of either the alkyl or acyl oxygen bond. The mode of ring opening is dependent on solution conditions and the type of nucleophile (2). The ability of β-lactones to acylate or alkylate nucleophiles suggests an unusual versatility for the modification of proteins.

The choice of the acid proteinase pepsin as the subject for initial studies was based on two facts. First, the methionine residues of pepsin have been reported to be resistant to alkylation by iodoacetate or iodoacetamide at pH 2.2 (3) while they are partially alkylated at pH 4.6 (4). β-Propiolactone (BPL) offers an alternative to the halo acetates in this respect.

Second, the active site of pepsin contains essential aspartate residues which might be alkylated by β-lactones yielding an esterified aspartate with concomitant formation of a new carboxylate moiety at the active site.

Porcine pepsin (Worthington Lot PM 36C867) 100 mg in 10 ml 0.1 M citrate (pH 3.0) was treated at 25°C with 50 µl portions of BPL at 30 min intervals for 2 hr. The mixture was stirred for an additional 3 hr at 25°C. The solution was divided into two equal portions. Excess reagent was removed from one portion by gel filtration on Sephadex G-25 eluting with 40 mM sodium citrate (pH 4.0). This material was used directly for kinetic analysis. The second portion was purified on a Sephadex G-25 column equilibrated with water followed by lyophilization. A control sample was treated in a manner identical to the above in the absence of BPL.

The BPL modified pepsin was inactive to the extent of 50% when assayed against hemoglobin (5). This decrease in activity is large when compared to previously reported effects of methionine modification by iodoacetate (4) and hydrogen peroxide (6).

The synthetic peptide substrate Cbz-His-Phe(NO_2)-Phe-OMe (7) was used to assess in more detail the effect of BPL modification on catalytic activity. Initial rates (<18% hydrolysis) were followed spectrophotometrically at 310 nm at four substrate concentrations. Plots of [S]/v vs. [S] (8) were employed to obtain the kinetic parameters K_M, V_{max}, and k_{cat}. For modified pepsin K_M remained essentially unchanged while k_{cat} was substantially decreased (Table 1). The 20-fold decrease in catalytic efficiency (k_{cat}/K_M) is due entirely to the decreased k_{cat}.

The extent of methionine modification was determined by performic acid oxidation (9), acid hydrolysis and amino acid analysis of the modified and lyophilized pepsin. The methionine sulfonium ion was recovered as methionine while unmodified methionine was found as methionine sulfone. The analysis indicated between 1 and 2 methionine residues were alkylated by BPL (Table 2). All other amino acids were found in the correct ratio.

Since previous work has indicated that methionine modification has at best only a small effect on pepsin activity it seemed reasonable to postulate that esterification of the active site by BPL had occurred. Treatment of the BPL modified enzyme with 1 M hydroxylamine at pH 9.0 (10) resulted in the incorporation of 0.29 moles hydroxamate per mole of pepsin as determined by the method of Bergmann and Segal (11). Treatment of the hydroxamate enzyme with fluorodinitrobenzene followed by the Lossen rearrangement and acid hydrolysis as described by Gross and Morell (10) resulted in the

formation of 0.35 moles 2,3-diaminopropionic acid per mole of pepsin. The control sample contained no detectable 2,3-diaminopropionate.

The marked drop in k_{cat} toward the peptide substrate and the trapping of an esterified aspartate residue in BPL modified pepsin suggests that alkylation of an active site aspartate occurred. It must be noted, however, that the modification of both methionine and aspartate residues makes interpretation of the kinetic results difficult. The demonstration that the esterified aspartate is definitely associated with the active site will have to await the determination of its location in the linear sequence of the protein.

We have recently found that 4-(p-nitrophenyl)-oxetan-2-one partially inactivates pepsin without modification of methionine residues. Also, an attempted alkylation of methionine 192 of chymotrypsin with the same reagent resulted in the apparent acylation of the active site serine 195.[1] It therefore seems that design and synthesis of active site directed β-lactones may yield new and versatile reagents for use in enzyme chemistry.

Acknowledgments

Partial support of this research by USPHS grant No. CA-17150 and NSF grant No. BMS 73-01568 (E.T.K.) and by USPHS Fellowship No. 1 F32 GM05516-01 (R.N.A.) is gratefully acknowledged.

[1] R.N. Armstrong and E.T. Kaiser, unpublished results.

Table 1: Hydrolysis of Cbz-His-Phe(NO_2)-Phe-OMe by Native and BPL-Modified Pepsin

Pepsin	K_M(m$\underline{M}$)	k_{cat}(sec^{-1})	k_{cat}/K_M (sec^{-1} m$\underline{M}$ $^{-1}$)
Native [a]	0.46	0.29	0.63
Control [b]	0.63	0.21	0.33
Modified [b]	0.56	0.0095	0.017

[a] Data obtained from reference 7. [b] Experiments were conducted at 37.0°C in 40 m$\underline{M}$ sodium citrate buffer (pH 4.0) containing 3.2% DMSO. [S] = 0.1 -0.5 m$\underline{M}$. [E] = 1.72×10^{-3} m$\underline{M}$ for control and 5.03×10^{-3} m$\underline{M}$ for modified enzyme.

Table 2: Methionine and Methionine Sulfone Content of Control and Modified Pepsins Before and After Performic Acid Oxidation

Pepsin	residues/mole Methionine	Methionine sulfone	Total
Control			
Acid Hydrolysis[a]	3.96	0.0	3.96
PAO[b]	0.0	4.01	4.01
Modified			
Acid Hydrolysis	3.83	0.0	3.83
PAO	1.57[c]	2.50	4.07

[a] 6 N HCl, 105°C, 24 hrs. [b] Performic acid oxidized followed by acid hydrolysis. [c] Reflects methionine sulfonium ion content.

REFERENCES

1. (a) M.A. Taubman and M.Z. Atassi, Biochem. J., 1968, 106, 829; (b) M.Z. Atassi, Immunochemistry, 1969, 6, 801; (c) M.Z. Atassi, J. Koketsu and A.F.S.A. Habeeb, Biochim. Biophys. Acta, 1976, 420, 358.
2. H.E. Zaugg, Org. React., 1954, 8, 305.
3. T.A.A. Dopheide and W.M. Jones, J. Biol. Chem., 1968, 243, 3906.
4. L.A. Lokshina and V.N. Orekhovich, Biokhimiya, 1964, 29, 346.
5. R.B. Chow and B. Kassell, J. Biol. Chem., 1968, 243, 1718.
6. K. Kido and B. Kassell, Biochem., 1975, 14, 631.
7. K. Inouye and J.S. Fruton, Biochem., 1967, 6, 1765.
8. G.N. Wilkinson, Biochem. J., 1961, 80, 324.
9. E. Schram, S. Moore and E.J. Bigwood, Biochem. J., 1954, 57, 33.
10. E. Gross and J.L. Morell, J. Biol. Chem., 1966, 241, 3638.
11. F. Bergmann and R. Segal, Biochem. J., 1956, 62, 542.

Received, 10th August, 1976