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Photochemical evidence for the participation of histidine in the active center of carboxypeptidase Aγ

Carboxypeptidase A has been shown to exist in at least four different forms, α, β, γ and δ (ref. 1). They apparently result from modifications in methods of isolation and have been the subject of many chemical and physical investigations. It should be emphasized that, although in some respects they are similar, considerable evidence is available which indicates significant chemical differences among these forms. We will present additional evidence of these chemical differences and evidence that histidyl residues are important for both esterase and peptidase activity of carboxypeptidase A_γ.

The technique of dye-sensitized photooxidation has been used to chemically modify carboxypeptidase A_γ. We have found that the γ form of the enzyme rapidly loses both peptidase and esterase activity when exposed to dye-sensitized photooxidation. The progressive loss of peptidase and esterase activity of the Rose Bengal or methylene blue sensitized systems is shown in Fig. 1. The kinetics of this inactivation do not appear to be first order and will require further investigation. In contrast, when the δ form of the enzyme is subjected to methylene blue sensitized photooxidation[2], the esterase activity increases 2-fold compared to the native state, demonstrating yet another difference between these enzymatic forms.

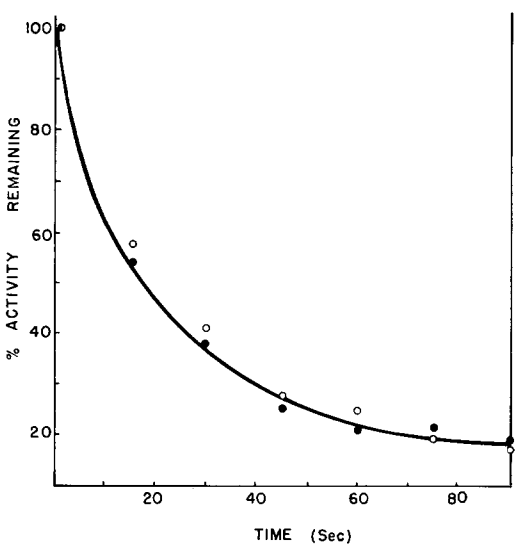

Fig. 1. Photooxidation of carboxypeptidase A_γ in 1.0 M NaCl, 0.1 M Tris, 0.10 mg/ml Rose Bengal, pH 7.5, at 3°. Protein concentration 0.28 mg/ml. 500-W light source focused through an f 1.6 lens was used. Samples were placed 4.3 cm from the lens. Esterase activity (○—○) determined spectrophotometrically by following $\Delta A_{254\ m\mu}$/min on a Gilford spectrophotometer. Esterase substrate was 10^{-3} M hippuryl-DL-phenyllactic acid in 0.025 M Tris, 0.5 M NaCl, pH 7.5, 25°. Peptidase activity (●—●) determined in identical manner using 10^{-3} M hippuryl-L-phenylalanine as substrate.

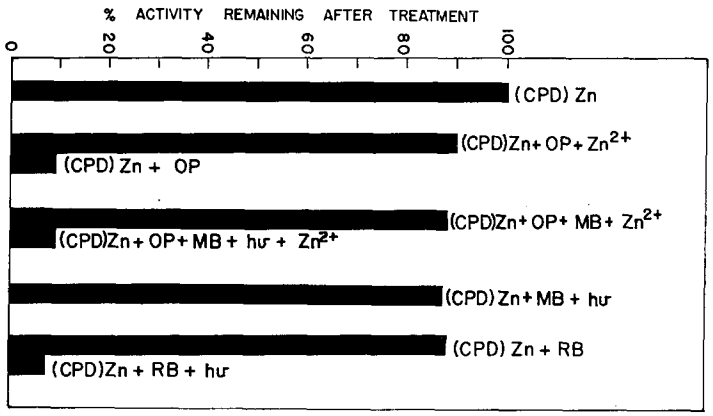

Fig. 2. Reagents were dissolved in 0.1 M Tris, 1.0 M NaCl, pH 7.0 at 0° with the following concentrations: methylene blue, 0.10 mg/ml; Zn^{2+}, 0.1 M; 1,10-phenanthroline, $2 \cdot 10^{-3}$ M; Rose Bengal, 0.10 mg/ml. Enzyme concentration, 0.02 mg/ml. A typical experimental run was prepared as follows: 0.25 ml of 1,10-phenanthroline buffer, 0.5 ml of dye containing buffer, 5 μl of carboxypeptidase A_γ (4.2 mg/ml), and 0.25 ml of 0.1 M Tris–1.0 M NaCl buffer (pH 7.0). 5 μl of 0.1 M Zn^{2+} buffer was added just prior to assay. Peptidase or esterase activities were determined as in Fig. 1. The light source was a 500-W projector, the sample placed 20 cm from the projector lens. Abbreviations: MB, methylene blue; OP, 1,10-phenanthroline; RB, Rose Bengal; *hν*, 30-min exposure to light; (CPD)Zn, carboxypeptidase A_γ.

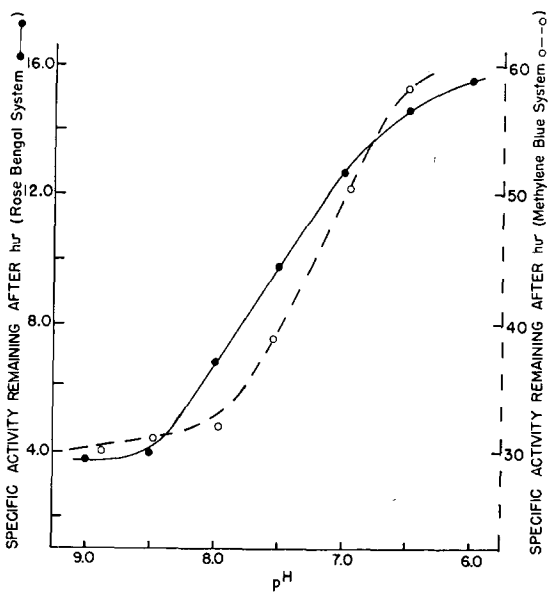

Fig. 3. The pH dependence of the methylene blue–1,10-phenanthroline sensitized photooxidation (○—○), determined as follows: 5 μl of a carboxypeptidase A_γ solution (4.03 mg/ml) was placed into duplicate tubes containing 1.0 ml of 0.1 M Tris, 1.0 M NaCl, $1 \cdot 10^{-3}$ M 1,10-phenanthroline, 0.19 mg/ml methylene blue buffer adjusted to the indicated pH values at 0°. One tube was kept in the dark, the second was exposed 10 min, at a distance of 20 cm, to a 500-W light source. 10 μl of a buffered 0.1 M $ZnCl_2$ solution was added to each tube and the peptidase activity determined. The pH dependence of the Rose Bengal sensitized system was done in a similar manner using 1.0 ml of 0.1 M Tris, 1.0 M NaCl, 0.10 mg/ml Rose Bengal buffer adjusted to the indicated pH values. The exposure time was cut to 5 min due to the more rapid loss of activity with this dye. Peptidase activity was measured as described in Fig. 1.

The data in Fig. 2 illustrate an interesting difference between the dyes Rose Bengal and methylene blue. Methylene blue requires the presence of a metal chelator in order to be an effective sensitizer, while Rose Bengal does not. Measurements of activity in the methylene blue–1,10-phenanthroline system required the addition of Zn^{2+} to reverse the loss of activity due to metal chelation[3]. Apocarboxypeptidase A_γ was prepared as described by VALLEE[4] and was found to be labile to photooxidation with methylene blue in the absence of the chelator. Since Rose Bengal does not inhibit the enzyme in the dark over extended periods of time, but rapidly inactivates the enzyme when exposed to light, it would appear that the dyes differ in their mechanisms of action.

The photooxidation of histidine has been shown to be pH dependent[5], the curve being sigmoidal shaped and having an inflection near pH 7.0. The amino acids tryptophan, tyrosine and methionine, which are also susceptible to photooxidation, do not show this pH dependence[5]. Thus, it is significant that carboxypeptidase A_γ, when photooxidized with Rose Bengal or methylene blue in the presence of 1,10-phenanthroline, shows a sigmoidal pH profile which is almost identical to that for the photooxidation of histidine (Fig. 3).

Amino acid analysis of photooxidized carboxypeptidase A_γ indicates that histidine is the only amino acid that is destroyed to any appreciable degree. Cysteine, tyrosine and methionine remain practically unaltered compared to control hydrolysates. The destruction of histidine by dye-sensitized photooxidation seems to follow an all-or-none pathway; that is, a 50% inactivated enzyme preparation has lost about 50% of the total histidine. This evidence implies that at least one residue is catalytically functional but does not exclude the possibility that more than one is involved.

Considerable indirect evidence has appeared in the literature which suggests the involvement of histidine in the mechanism of action of the enzyme. The pH-rate profile of peptide hydrolysis has the normal bell-shaped curve with an inflection at pH 6.7 (ref. 2), commonly attributed to histidine. Studies employing 5-diazo-1H-tetrazole have suggested the possible involvement of histidine in the catalytic function of the enzyme[6]. Most significant, perhaps, is the X-ray structure of carboxypeptidase A_α (ref. 7), in which histidine has been shown to be a zinc ligand.

In summary, we feel that these photochemical studies provide substantial chemical evidence for histidyl involvement in the catalytic function of carboxypeptidase A_γ. We have demonstrated loss of both peptidase and esterase activity by photochemical techniques. Since simultaneous loss of both activities has never resulted from anything other than the removal of the zinc from its binding site, and since histidine functions as a zinc ligand in the α form, we favor the inclusion of at least one of the photochemically labile histidyl residues as a zinc ligand in carboxypeptidase A_γ.

It should be emphasized that alternate explanations of the function of histidyl or histidyl residues would be consistent with the data. One or several histidines could be acting directly in the catalytic function as general acid–base nucleophiles or as substrate binding contact amino acids. Because of the apparent complexity of the dye-sensitized reaction, photooxidation of one or more histidines might allow subsequent photooxidation of the remaining histidines in the molecule. Thus, a photochemically labile histidine could be essential in maintaining the integrity of the native enzyme. Studies are now in progress in an attempt to further delineate the function(s) of histidine in carboxypeptidase A_γ.

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