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Carboxypeptidase A. Quantum Yields on Ultraviolet Irradiation*

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ABSTRACT: Ultraviolet irradiation (2537 A) inactivates carboxypeptidase A with an over-all quantum yield, Φ_{total}, of 4.9×10^{-3}. Three distinct processes have been shown previously to be responsible for the inactivation of carboxypeptidase: loss of zinc, alteration of a tyrosine at the active center, and changes in structure resulting in denaturation. The quantum yields of destruction for each of these processes have been determined and are 1.6×10^{-3}, 5×10^{-4}, and 2.8×10^{-3}, respectively. Φ_{total} is smaller than the value predicted, confirming that only some of the absorbing amino acids are essential for activity. Comparison of the individual quantum yields obtained with those calculated shows that the quantum yield of inactivation of an enzyme can only be predicted exactly when the nature and number of amino acid residues directly or indirectly involved in enzymatic activity can be established.

McLaren and Luse (1961a,b, 1963) have proposed that the quantum yield of inactivation of any enzyme by ultraviolet irradiation, Φ_{enzyme}, can be calculated by summation of all the quanta contributing to inactivation through effects on different and independent residues. The expected over-all quantum yield of inactivation can be obtained by substitution in eq 1

$$\Phi_{enzyme} = \frac{\Sigma^{i} n_i \epsilon_i \phi_i}{\epsilon_e} \qquad (1)$$

where n is the number of residues of the amino acids (i) per molecule of enzyme, ϵ_i the molar absorptivity, ϕ_i the quantum yield for the destruction of each amino acid, and ϵ_e the molar absorptivity of the enzyme. Using eq 1, Φ_{enzyme} has been calculated for a number of enzymes, including carboxypeptidase A, and these values have been compared with those observed experimentally (Luse and McLaren, 1963; McLaren and Hidalgo-Salvatierra, 1964). In this calculation of Φ_{enzyme} all amino acids absorbing at the wavelength of irradiation are considered equivalent. Not all of the amino acid residues of enzymes are equivalent or essential for activity, however, an aspect of the problem which has been discussed in regard to ultraviolet irradiation of proteins (McLaren and Shugar, 1964). Augenstein and co-workers (Augenstein and Ghiron, 1961; Augenstein and Riley, 1964) have stressed the importance of the disruption of specific disulfide and hydrogen bonds consequent to the irradiation of certain enzymes.

In the course of recent studies of the effect of ultraviolet irradiation on carboxypeptidase A (Piras and Vallee, 1966a,b) a new value for the quantum yield of inactivation of this enzyme was obtained. The observed decrease of peptidase activity could be attributed to at least three distinct processes: loss of zinc, specific alteration of a tyrosyl residue at the active center, and disruption of protein structure, accompanied by destruction of tryptophanyl and histidyl residues. Thus, the over-all quantum yield of inactivation of carboxypeptidase, Φ_{total}, can be considered to be the sum of the quantum yields for each of these processes.

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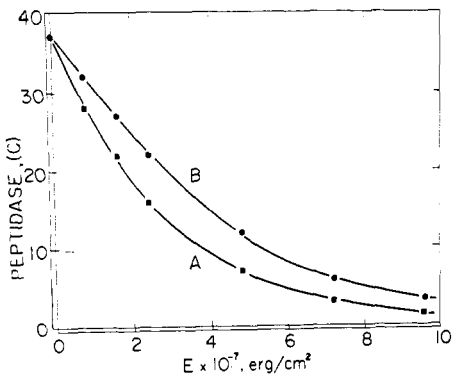

FIGURE 1: Effect of ultraviolet irradiation (2537 A) on peptidase activity of carboxypeptidase (4×10^{-5} M) in 1 M NaCl–0.1 M Tris-HCl buffer (pH 7.5), at 4°. Curve A: activities based on original protein concentration. Curve B: activities based on zinc still bound to the protein after extensive dialysis. Assays were carried out as previously described (Piras and Vallee, 1966a).

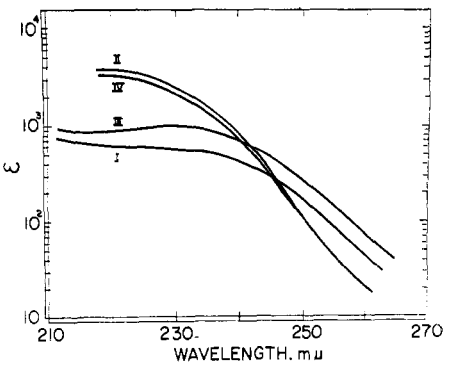

FIGURE 2: Absorption spectra of β-mercapoethanolamine (I), Zn–β-mercaptoethanolamine (II), cysteine (III), and Zn–cysteine (IV). The ligands (5×10^{-4} M) and zinc (1×10^{-2} M) were in 0.1 M Tris-HCl buffer (pH 7.4), 25°.

$$\Phi_{total} = \Phi_{Zn} + \Phi_{Tyr} + \Phi_{den} \qquad (2)$$

The present communication compares the quantum yields observed for each of these processes in carboxypeptidase A with those expected from McLaren's formulation, here applied in a more restrictive manner, *i.e.*, only to those amino acids and bonds of the protein which have been found reactive experimentally to ultraviolet irradiation and, hence, presumably essential for the preservation of enzymatic activity.

Materials and Methods

Twice-recrystallized bovine pancreatic carboxypeptidase A was obtained from Worthington Biochemical Corp., Freehold, N. J. The enzyme suspension was centrifuged, and the crystals were washed three times with water before dissolving in 1 M NaCl–0.01 M Tris-HCl (pH 7.5). The zinc:protein ratio was 0.97–1.03 g-atoms/mole, based on a molar absorptivity ϵ_{278} 6.42×10^4 M^{-1} cm^{-1}; β-mercaptoethanolamine (Evans Chemicals, Inc.) and cysteine (Fisher Scientific Co.) were used without further purification.

Ultraviolet irradiation and measurements of enzymatic activity, zinc, and protein concentration were performed as previously described (Piras and Vallee, 1966a). A Cary Model 11 recording spectrophotometer was used to obtain continuous spectra, with 1-cm cells and at room temperature. Gel filtration was carried out with a 1.0×100 cm column of Sephadex G-75 (Pharmacia, Uppsala) equilibrated with 1 M NaCl–0.01 M Tris-HCl buffer (pH 7.5) at a flow rate of 0.3 ml/min. A 2-ml sample was applied, and 2-ml fractions were collected automatically and used for protein, zinc, and peptidase activity determinations. On gel filtration, irradiated carboxypeptidase exhibits three peaks, two of which (fractions I and II) have R_F values higher than that of the native enzyme (fraction III) (Piras and Vallee, 1966b).

Results

The effect of ultraviolet irradiation on the peptidase activity of carboxypeptidase A is shown in Figure 1. Curve A indicates the loss of peptidase activity, based on the initial protein concentration of the total irradiation mixture. The quantum yield is 4.9×10^{-3}, representing the over-all yield of inactivation due to ultraviolet irradiation, Φ_{total}, irrespective of the mechanism leading to the loss of activity.

Irradiation of carboxypeptidase results in loss of zinc from the enzyme (Piras and Vallee, 1966a). Curve B of Figure 1 represents the peptidase activity calculated on the basis of the zinc still bound to the protein after irradiation; hence, it reflects the loss of activity owing to the effect of ultraviolet irradiation on all parameters other than the zinc content. The corresponding quantum yield of inactivation, $\Phi_{Zn_{cor}}$, is 3.3×10^{-3}; that owing to zinc alone, $\Phi_{Zn} = 1.6 \times 10^{-3}$, represents the difference, $\Phi_{total} - \Phi_{Zn_{cor}}$.

The zinc atom of carboxypeptidase is bound to a sulfur–nitrogen ligand site of the enzyme (Vallee, 1964). Data on the quantum yield of destruction and molar absorptivity of suitable models for such a binding site are not available. Hence, absorption spectra for cysteine and β-mercaptoethanolamine, two ligands that can form S–Zn–N complexes, were obtained (Figure 2). The coefficients of absorptivity, ϵ_{2537}, are 120 and 200, respectively.[1] For both agents, addition of excess Zn^{2+}, sufficient to ensure complete formation of the 1:1 complexes, decreases ϵ_{2537} to 50.

[1] The ionized form of a sulfhydryl group absorbs maximally at 232–238 mμ (ϵ $4–6 \times 10^3$) (Benesch and Benesch, 1955). The absorptivities here observed at 2537 A and pH 7.4 may be assumed to be owing to the ionized fraction of the thiol.

Gel filtration of ultraviolet-irradiated carboxypeptidase on Sephadex G-75 reveals three peaks (Piras and Vallee, 1966b), one of them, fraction III, emerging from the column in the same position as the native enzyme. The protein content of this fraction decreases as a function of irradiation (Figure 3A), and the quantum yield is 4.5×10^{-3}. The only modification detected in this fraction is the destruction of a tyrosyl residue, allowing for the partial loss of zinc (*vide supra*), which results in the decrease of peptidase and the increase of esterase activity (Piras and Vallee, 1966b). The quantum yield of inactivation solely due to tyrosine modification, $\Phi_{Tyr} = 5 \times 10^{-4}$, is obtained when peptidase activity is based on the measured zinc content of fraction III (Figure 3B).

Finally, Φ_{den}, the quantum yield of denaturation, the third term of eq 2, is found by difference $\Phi_{total} - (\Phi_{Zn} + \Phi_{Tyr}) = \Phi_{den} = 2.8 \times 10^{-3}$. These quantum yields from experimental values and those calculated using eq 1 and the values of Φ_i and ϵ_i (Luse and McLaren 1963) are compared in Table I.

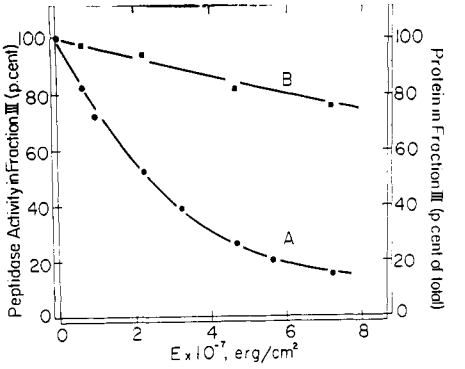

FIGURE 3: Effect of ultraviolet irradiation (2537 A) of carboxypeptidase (4×10^{-5} M) on protein content (A) and peptidase activity (B) of fraction III. Curve A: the amount of protein found in fraction III after separation on Sephadex G-75 (see text) as estimated from absorbance at 278 mμ. Curve B: peptidase activity of fraction III, based on the amount of zinc enzyme present as determined by absorbance and zinc analyses.

TABLE I: Irradiation of Carboxypeptidase with Ultraviolet Light (2537 A): Observed and Calculated Quantum Yields.

	Quantum Yield × 10^3	
	Found	Calcd[a]
Φ_{total}	4.9	10.0[b]
$\Phi_{Zn,tot}$	3.3	—
Φ_{Zn}	1.6	—
Φ_{Tyr}	0.5	0.05[c]
Φ_{den}	2.8	2.2[d]
		3.3[e]

[a] Using eq 1 and the values for Φ_i and ϵ_i given by Luse and McLaren (1963). ϵ_{254} for carboxypeptidase is 2.6×10^4. [b] The chromophores employed in the calculation are: His_8, Phe_{15}, Trp_8, and Tyr_{19} (Bargetzi *et al.*, 1963). [c] Assuming two tyrosyl residues at the active center (Vallee, 1964). [d] Assuming two tryptophanyl residues. [e] Assuming three tryptophanyl residues.

Discussion

Calculation of Φ_{enzyme} for carboxypeptidase A by means of eq 1 as proposed by McLaren and based on the presently accepted amino acid composition (Bargetzi *et al.*, 1963) yields a value of 10×10^{-3} (Table I). This quantum yield is twice that determined experimentally, *i.e.*, 4.8×10^{-3} (Piras and Vallee, 1966a),[2] and is larger by one order of magnitude than that previously found (McLaren *et al.*, 1953). Similarly, the quantum yields for a number of proteins calculated using eq 1, and based on all absorbing chromophores, are higher than those observed (McLaren and Hidalgo-Salvatierra, 1964). However, better agreement would be expected if only those amino acids were considered which are critical to activity, either directly or indirectly (McLaren and Shugar, 1964; Augenstein and Ghiron, 1961; Augenstein and Riley, 1964).

However, discrepancies are still observed even when this factor is taken into consideration. Assuming that the extinction coefficients and quantum yields of destruction derived from models of individual amino acids pertain precisely to proteins, the difference could be due to an energy transfer mechanism.

Unfortunately, the observed quantum yield of inactivation due to zinc loss in carboxypeptidase is not readily compared to a suitable model. At best, a quantum yield can be inferred from the molar absorptivities of cysteine or β-mercaptoethanolamine and their 1:1 zinc complexes. The molar absorptivities of these zinc complexes are quite similar to that of zincthionein (Kägi and Vallee, 1961), a protein especially suitable for comparisons owing to its large number of S–Zn chromophores and very low content of aromatic amino acids. Assuming that the molar absorptivities of the models reflect the characteristics of the zinc binding site of carboxypeptidase, a quantum yield of 0.79 would have to be assumed for the destruction of an S–Zn–N linkage to account for $\Phi_{Zn} = 1.6 \times 10^{-3}$ (Table I). Similarly high quantum yields for specific cystinyl residues of trypsin and ribonuclease have been reported, and their significance has been discussed (Augenstein and Ghiron, 1961; Augenstein and Riley, 1964; Siebert *et al.*, 1965). Since the protein structure of carboxypeptidase is altered extensively (Piras and Vallee,

[2] This value better fits the finding of McLaren *et al.* (1953) that for a number of enzymes Φ_{enzyme} is proportional to the reciprocal of their molecular weights.

1966b), the possibility cannot be dismissed, of course, that zinc loss is not due primarily to energy absorption or transfer to the S–Zn–N linkage, but rather is the consequence of general disruption of protein structure (Piras and Vallee, 1966b).

The quantum yields for the two enzymatically active tyrosines of carboxypeptidase (Vallee, 1964), calculated by means of eq 1 from data on amino acids irradiated in solution (Luse and McLaren, 1963), are one order of magnitude lower than Φ_{Tyr} observed (Table I). There are several possible explanations. Thus, the quantum yield of destruction for these particular tyrosyl residues of carboxypeptidase may be larger than that found for the free amino acid (Luse and McLaren, 1963). The known high chemical reactivity of these tyrosyl residues of carboxypeptidase (Vallee, 1964) would tend to support such an hypothesis. Alternately, the experimental value could reflect an energy-transfer mechanism. Further, small structural alterations might exist, even though the gross molecular parameters remain unaltered (Piras and Vallee, 1966b). Any one of these alternatives or their combination would render the quantum yield larger than that expected for tyrosine modification alone.

The third term of eq 2, Φ_{den}, can be calculated using the values for the quantum yield of destruction and molar absorptivity for tryptophan at pH 7 (Luse and McLaren, 1963). Modification of either two or three tryptophanyl residues could account for the observed value of Φ_{den}, consistent with the results of amino acid analyses obtained on native and irradiated carboxypeptidase (Piras and Vallee, 1966a). The need for a Φ_{den} term has been recognized recently by McLaren (1966).

Exposure of carboxypeptidase to hydrogen peroxide almost completely mimics the effects of ultraviolet irradiation (Piras and Vallee, 1966a,b). Thus, the question might be raised whether the consequences of ultraviolet irradiation of carboxypeptidase could be due to a free radical mechanism, rather than to a primary photochemical effect, as here assumed. However, Φ_{enzyme} is independent of the presence of oxygen (Piras and Vallee, 1966a); therefore, the similarity in the enzymatic patterns of the two modifications is probably owing to their effects on the same residues rather than to a common mechanism. An independent check on the validity of the photochemical scheme can be obtained by comparing the sum of Φ_{Zn} plus Φ_{Tyr} with the quantum yield calculated from the disappearance of protein in fraction III, 4.5×10^{-3} (Figure 3A). This is in good agreement with 4.4×10^{-3}, the sum of Φ_{Zn} plus Φ_{den}, each obtained independently. This is consistent with the fact that the new fractions found on gel filtration after ultraviolet irradiation of carboxypeptidase have both lost most of their zinc and are denatured (Piras and Vallee, 1966b). Furthermore, if, as postulated in eq 2, the over-all quantum yield is the sum of the individual quantum yields described, the rate of loss of enzymatic activity should be equal to or greater than the rates of any of the individual processes observed. Comparison of the data in Figures 1 and 3 shows this to be the case. Since carboxypeptidase does not contain S–S bridges (Bargetzi *et al.*, 1963) these studies do not permit any examination of alternative schemes of inactivation involving their role (Augenstein and Ghiron, 1961; Augenstein and Riley, 1964).

Thus, the quantum yield of inactivation of an enzyme, such as carboxypeptidase, can be calculated exactly only when the nature and number of those amino acid residues are known which are affected either by irradiation and involved directly in function, or involved indirectly in maintaining the native structure of an enzyme. When such data are available, calculations and experiment show good agreement. Further, when the different pathways leading to inactivation can be segregated experimentally, as in the carboxypeptidase A system, comparison of the calculated and experimental values may be used to infer the mechanism underlying the nature of the photochemical inactivation. Thus the results of chemical modifications (McLaren and Shugar, 1964; Augenstein and Ghiron, 1961; Augenstein and Riley, 1964; Siebert *et al.*, 1965) together with the approach proposed by McLaren and co-workers (McLaren and Luse, 1961a,b; Luse and McLaren, 1963) can account for the details which result in inactivation of an enzyme such as carboxypeptidase by ultraviolet light.

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Bromopyruvate Inactivation of 2-Keto-3-deoxy-6-phosphogluconic Aldolase. I. Kinetic Evidence for Active Site Specificity*

H. Paul Meloche

ABSTRACT: 2-Keto-3-deoxy-6-phosphogluconic aldolase of *Pseudomonas putida* strain A 3.12 catalyzes the reversible condensation of pyruvate and D-glyceraldehyde 3-phosphate as well as the exchange of the methyl hydrogens of pyruvate with protons of water. Studies were undertaken to demonstrate an active site amino acid residue involved in pyruvate α-hydrogen activation by use of the pyruvate analog monobromopyruvic acid. Bromopyruvate was found to inactivate the aldolase and the inactivation was first order with respect to the remaining active enzyme. Steady-state conditions were assumed to derive kinetic expressions for enzyme–inactivator complex formation and for substrate–inactivator competition. The rate of inactivation of the aldolase was proportional to bromopyruvate at low concentration and constant at high bromopyruvate concentration. The concentration of bromopyruvate giving the half-maximum rate of inactivation was 1 mM and the rate observed at infinite bromopyruvate concentration was equivalent to an apparent first-order rate constant of 0.0115 sec^{-1} at pH 6.0 and 24.5°. Pyruvate and 2-keto-3-deoxy-6-phosphogluconate protected competitively, but D-glyceraldehyde 3-phosphate had little or no effect on the bromopyruvate inactivation rate.

The apparent enzyme–pyruvate dissociation constant at pH 6.0 in the range 0.126–0.148 mM was obtained from protection experiments. Studies with radioactive bromopyruvate showed that two carboxyketomethyl residues were stably incorporated per mole of enzyme inactivated. This value agrees with the number of pyruvates bound when this enzyme is treated with $NaBH_4$. The results are consistent with the occurrence of a basic amino acid residue adjacent to the methyl carbon of pyruvate that is bound to the active site of the enzyme.

The enzyme KDP-gluconic[1] aldolase catalyzes the reversible condensation of pyruvate and D-glyceraldehyde 3-phosphate (Meloche and Wood, 1964a). The aldolase purified from *Pseudomonas putida* (formerly *fluorescens*) A.3.12 extracts was crystallized and found to have a molecular weight of approximately 90,000 (Meloche and Wood, 1964b). There is no evidence that the enzyme requires metal ions for activity. In the presence of borohydride and labeled pyruvate, 2 moles of pyruvate is stably fixed per mole of enzyme with virtually complete loss of activity (Meloche and Wood, 1964b), suggesting that the enzyme molecule contains two active sites. The data of Ingram and Wood (1965) are consistent with an azomethine occurring between the carbonyl carbon atom of pyruvate and the ϵ amino of active site lysine being reduced by borohydride.

One of the early steps in enolate–anion reactions is carbanion formation at the carbon atom adjacent to the carbonyl. This anion generation results from the loss of a proton. Thus, among enzymes catalyzing reactions that involve carbanion generation (*e.g.*, aldolases, isomerases, and β-decarboxylases), one might expect to find an active site basic amino acid residue involved in substrate deprotonation. This in an aldolase would contribute to the over-all reaction by helping tautomerize the azomethine to the enamine form as shown in Figure 1. A basic amino acid residue having this function in the active site of KDP-gluconic aldolase could be alkylated by an analog of pyruvate in which carbon atom three is electrophilic, such as monobromopyruvic acid. Alkylation and identification of such an amino acid would contribute to the over-all knowledge of proton-transfer reactions, as well as provide a possible means of bridging active site pep-

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[1] Abbreviations used: KDP-gluconic(ate), 2-keto-3-deoxy-6-phosphogluconic(ate); CKM, carboxyketomethyl; NADH, reduced nicotinamide–adenine dinucleotide.