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Modification of Carboxyl Groups in Bovine Carboxypeptidase A. I. Inactivation of the Enzyme by *N*-Ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K)*

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ABSTRACT: Bovine carboxypeptidase A is rapidly inactivated by Woodward's reagent K. The enzyme can be protected against inactivation by β-phenylpropionate and other competitive inhibitors. Up to 50% inactivation, the loss of enzymatic activity is proportional to the incorporation of reagent. After correcting for modification of nonfunctional sites, linear extrapolation to zero activity reveals the incorporation of approximately 1 mole of reagent/mole of protein. Kinetic studies demonstrate a rate-limiting step in the inhibition reaction, resulting in the formation of a reversible [enzyme·reagent] complex. The calculated $K_{I,app}$ is $3.77 \pm 0.1 \times 10^{-2}$ M at pH 6.4. The rate of inactivation is pH dependent with an extrapolated pK of approximately 7. When compared to other kinetic studies, these data strongly suggest that the same group which is inactivated by reagent K is essential to the mechanism of action of the enzyme.

As part of a continuing effort to elucidate the relationship between the chemical structure and enzymatic function of bovine carboxypeptidase A, the chemical modification of carboxyl groups was investigated. The three-dimensional model described by Lipscomb and coworkers (1968) and the recent elucidation of the primary structure of the enzyme (Bradshaw *et al.*, 1969) reveal the presence of two carboxyl groups in the active site of the enzyme, namely, Glu_{72} and Glu_{270}. The former is one of the three zinc ligands and the latter is thought to be important in the catalytic mechanism by serving either as a nucleophile or as a general base.

Recently a new class of reagents useful in the chemical modification of protein carboxyl groups has been described (Bodlaender *et al.*, 1969). One of these, Woodward's reagent K, incorporates in its chemical structure some of the features common to normal substrates of carboxypeptidase A, *i.e.*, an aromatic function and a negative charge, and could therefore be expected to have some affinity for the active center. Preliminary investigations showed that the enzyme was rapidly inactivated by this reagent (Pétra and Neurath, 1970). This paper describes an extensive study of this inhibition.

Experimental Section

Materials

Carboxypeptidase A (Anson) was purchased from Worthington Biochemical Corp. and chromatographically purified according to Pétra and Neurath (1969). CPA[1] (Anson), CPA_γ^{Val}, and CPA_γ^{Leu} were used in this work.

Carbobenzoxyglycyl-L-phenylalanine (CGP) and sodium hippuryl-DL-phenyllactate (HPLA) were purchased from Cyclo Chemical Co. and Fox Chemicals, respectively. β-Phenylpropionic acid, Baker Analyzed reagent, was recrystallized from an ethanol–water mixture. Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate) was purchased from Aldrich. [^{14}C]*O*-Methylhydroxylamine was purchased from New England Nuclear and diluted with recrystallized, unlabeled reagent obtained from City Chemical Co. Omni-

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[1] The following abbreviations are used: CPA, carboxypeptidase A; CPA_γ^{Val} and CPA_γ^{Leu} are the valine and leucine allotypes of the γ form of carboxypeptidase A (Pétra *et al.*, 1969); CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactate; K, Woodward's reagent K; K-CPA, derivative produced by the reaction of Woodward's reagent K with CPA; M-CPA, methoxamide derivative of K-CPA; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.

fluor was purchased from New England Nuclear. All other chemicals were reagent grade and were recrystallized when necessary.

Methods

Kinetic Measurements. Enzyme stock solutions were prepared by dissolving the crystals in 5 M NaCl–0.005 M potassium phosphate (pH 7.5) at 0°, and diluting with distilled water to the proper concentration. Protein concentration was estimated at 278 nm with the Zeiss PMQ-II spectrophotometer using $\epsilon_{278} = 6.49 \times 10^4\ M^{-1}\ cm^{-1}$ (Bargetzi *et al.*, 1963) or by amino acid analysis in the presence of a norleucine standard for the calculation of extinction coefficients. The standard assays for peptidase (CGP) and esterase (HPLA) activities have been previously described (Whitaker *et al.*, 1966; Bargetzi *et al.*, 1963). Peptidase activities and absorption spectra were measured using Cary Model 16 and Model 15 spectrophotometers, respectively. Esterase activity was measured by titration in the pH-Stat (Radiometer TTT-1). The pseudo-first-order kinetic constants of inhibition, k_{app}, were calculated from the slope of plots of log of per cent of activity remaining *vs.* time. The first-order rate constant of inhibition, k_2, as well as the steady-state constant, $K_{I,app}$, were calculated from the reciprocal plot $1/k_{app}$ *vs.* 1/[I] as described below (Kitz and Wilson, 1962; Shaw and Glover, 1970).

Amino acid analyses were performed by the accelerated amino acid analyzer system described by Spackman (1967). Samples were hydrolyzed for 22 hr at 110° in evacuated tubes. Ethylamine was determined by use of a 6.0-cm column of AA-27 Beckman spherical resin at 51° with a buffer and ninhydrin flow rate of 68 and 34 ml per hr, respectively. The buffer used was 0.21 N sodium citrate (pH 5.20). The constant for ethylamine was calculated from the value reported by Bodlaender *et al.* (1969)[2] after correcting for the difference in the ninhydrin constants of the basic amino acids. The ratio of ninhydrin constants of lysine:histidine agreed within 3% of that reported by these workers. The relatively low color value of ethylamine necessitated the application of large amounts of protein hydrolysate to the short column (3–4 mg/analysis).

Reaction of CPA with Woodward's Reagent K (K-CPA). An enzyme stock solution was prepared by dissolving the crystals in 5 M NaCl–0.005 M potassium phosphate (pH 7.5) at 0° and diluting with distilled water to a protein concentration of 35 mg/ml in 1 M NaCl. This solution was stable for at least 2 weeks at 4°. The pseudo-first-order rate constants for the hydrolysis of CGP and the pseudo-zero-order rate constant for the hydrolysis of HPLA were, respectively, 20 and 120 sec^{-1} (Pétra, 1970). An aliquot of enzyme stock solution was added to a given volume of 1 M NaCl–0.005 M potassium phosphate (0.64 mg/ml, 1.85×10^{-5} M) and allowed to equilibrate for 10 min at the desired pH at 25°. A small aliquot of freshly prepared Woodward's reagent K (stock solution: 120 mg/ml in 10^{-3} N HCl, equivalent to 0.47 M) was quickly added with stirring, resulting in a final concentration of 1.9×10^{-3} M of reagent in the reaction mixture. The pH was maintained constant by the addition of 0.033 N NaOH to 10 ml of the reaction mixture. (The normality of the base was increased when higher concentrations of the reagent or larger reaction volumes were used.) At prescribed times, aliquots (10–80 μ were removed to measure enzymatic activity using standard esterase (HPLA) and peptidase (CGP) assays. Other samples were removed from the reaction mixture and precipitated with 100 μl of 98% formic acid. The precipitates were thoroughly washed with 1 M NaCl adjusted to pH 3 with 98% formic acid in order to ensure the removal of excess Woodward's reagent K as well as breakdown products prior to amino acid analysis. Spectral analyses were carried out on the modified protein by terminating the reaction at prescribed times by the addition of 13.3 M sodium formate (pH 7.0) equivalent to one-tenth of the total volume of the reaction mixture. The solution (5–10 ml) was passed through a 1.5 × 90 cm column of Sephadex G-25 equilibrated with 1 M NaCl–0.05 M potassium phosphate (pH 6.4) at 4°. When large volumes of reaction mixture were involved, as in the case of subsequent reactions with a nucleophile (see below), the solution was concentrated using a Diaflo (Amicon Corp.) equipped with a UM-10 or PM-10 membrane (Pétra and Neurath, 1969). K-CPA was prepared in the presence of 2×10^{-3} M β-phenylpropionate as described above.

Nucleophilic Displacement of K-CPA by Methoxamine

Preparation of Methoxamide-CPA. Nucleophilic displacement of the label with methoxamine was performed by the following methods.

METHOD 1. The enzyme was first reacted with Woodward's reagent K as described above (40- to 100-ml reaction). The reaction was terminated with 13.3 M sodium formate (pH 7.0) and immediately cooled to 0°. The protein solution was concentrated to about 10 ml (4–5 mg/ml) at 4°[3] in the Diaflo pressure cell and fractionated at 4° on a 1.5 × 90 cm column of Sephadex G-25 equilibrated with 1 M NaCl–0.01 M potassium phosphate (pH 6.4). The protein fraction was concentrated in the Diaflo to about 4 ml (8–10 mg/ml), centrifuged to remove turbidity, and added to 4 ml of 2 M methoxamine at pH 6.4. The reaction was continued for a prescribed time and temperature, depending on the experiment. The solution was then centrifuged[4] and passed again through the Sephadex G-25 column prior to analysis for radioactivity, enzymatic activity, absorption, and ethylamine.

METHOD 2. This method was similar to method 1 except that the first gel filtration on Sephadex was omitted. Excess reagent K and other small molecular weight contaminants were removed in the Sephadex G-25 column after reaction. This procedure decreased by 8–10 hr the time K-CPA remains in solution (see Results).

METHOD 3. In this method, both Sephadex G-25 columns were omitted. At the end of the nucleophilic displacement reaction, the protein was precipitated by adding dropwise with stirring 98% formic acid to pH 3. The precipitate was washed ten times with 8-ml aliquots of 1 M NaCl adjusted to pH 3 with 98% formic acid to remove excess reagent and excess [^{14}C]methoxamine when used. (The presence of salt is important to keep the protein insoluble.) The precipitated protein was suspended in 30 ml of water, dialyzed exhaustively against water, and lyophilized. The powder obtained was used subsequently for peptide isolation (Pétra and Neurath, 1971).

Radioactivity Measurements. Aliquots (10–50 μl) of radioactive solutions were added to vials containing 10 ml of scintil-

[2] The author thanks Dr. Elliott Shaw for sending him a preprint of this paper.

[3] Although large amounts of modified protein are lost under these conditions, no better method for concentrating carboxypeptidase solutions could be found. Lyophilization yields insoluble and inactive enzyme.

[4] M-CPA becomes progressively insoluble and the turbidity must be removed before gel filtration. On the other hand, when K-CPA is prepared in the presence of β-phenylpropionate, the corresponding M-CPA derivative is soluble.

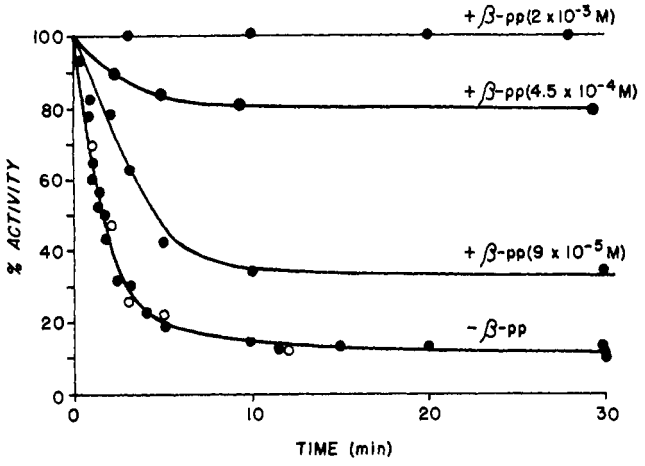

FIGURE 1: Rate of inactivation of carboxypeptidase A (Anson) (1.84×10^{-5} M) by Woodward's reagent K (1.89×10^{-3} M) in 1 M NaCl–0.005 M potassium phosphate (pH 6.4) at 25°, in the presence and absence of β-phenylpropionate (β-pp). (●) Esterase activity using HPLA as substrate; (○) peptidase activity using CGP as substrate. The rate of inactivation was the same when Tris or Veronal was used as buffer salts.

TABLE I: Kinetics of Inactivation of Carboxypeptidase A by Woodward's Reagent K.[a]

pH	[K] 10^3 (M)	k_{app}[b] (min^{-1})
6.2	1.89	0.178
6.3	1.89	0.256
6.4	1.89	0.308
6.4	2.4	0.362
6.4	2.9	0.426
6.4	3.3	0.495
6.4	4.8	0.704
6.6	1.89	0.422
7.0	1.89	0.99
7.3	1.89	1.31
7.6	1.89	1.47

[a] [E] = 1.85×10^{-5} M, 25°. Buffer: 1 M NaCl–0.005 M potassium phosphate. [b] Calculated from Figure 3 and eq 7 and 8.

lation fluid (500 g of naphthalene and 32 g of Omnifluor/4 l. of *p*-dioxane) and counted in a Packard Model 3003 scintillation spectrometer. Correction for quenching was applied by counting vials, each containing 10–50 μl of solvent plus a known amount of radioactivity taken from a [^{14}C]toluene standard.

Results

Reaction of Woodward's Reagent K with Carboxypeptidase A (Anson). The esterase and peptidase activities of carboxypeptidase A (Anson), CPA_γ^{Val}, and CPA_γ^{Leu} were rapidly abolished by reagent K. Figure 1 shows a typical inhibition profile of CPA (Anson) at pH 6.4 and 25° using a 100-fold excess of reagent. Forty-six per cent of activity remained after 2.5-min reaction time. Pseudo-first-order rate constants of inhibition, k_{app}, are given in Table I. Identical values were obtained for CPA_γ^{Val} and CPA_γ^{Leu}. Reproducibility of k_{app}, however, depended greatly on maintaining a constant pH during the inhibition reaction. The activity remaining after 30 min could be abolished by adding another aliquot of reagent. The rate of inactivation decreased in the presence of the competitive inhibitor β-phenylpropionate which fully protected the enzyme at concentrations of 2×10^{-3} M. The order of protection against inactivation by other reversible inhibitors was inverse to the order of their K_I values, as shown in Figure 2.

Stability of Woodward's Reagent K. The stability of the reagent was measured as a function of pH. A series of reagent solutions (0.47 M) was allowed to remain at pH 3, 4, 5, 6, and 7 at 25°. After 2 min, aliquots were removed from each solution and added to active enzyme solutions (1.85×10^{-5} M pH 6.4) to measure the rate of inhibition. Although the reagent was found to be completely stable at pH 3, it was rapidly destroyed above this pH. It was not possible to calculate a second-order rate constant of inhibition at pH 3 because the enzyme is not stable at these acidic conditions. Consequently, the actual concentration of reagent at any time during a particular inhibition reaction at pH's higher than 3 could not be determined by this method. Nevertheless, using the standard inhibition conditions described in Figure 1, the reagent had lost about 60, 70, and 90% of its efficiency to inhibit CPA after being exposed to pH 4, 6, and 7 for 2 min at 25°, respectively. It was therefore important to use a large excess of reagent over enzyme in order to obtain pseudo-first-order kinetics of inhibition as shown in Figure 3.

Kinetics of Inactivation. EVIDENCE FOR A REVERSIBLE [ENZYME·REAGENT] COMPLEX. Evidence for the existence of a rate-limiting step in the irreversible inhibition of certain enzymes by substrate-like reagents has been demonstrated. For example, the kinetics of cholinesterase inhibition by certain methanesulfonic acid esters (Kitz and Wilson, 1962) and the kinetics of inhibition of trypsin by TLCK (Shaw and Glover, 1970) reveal the presence of a reversible complex between the inhibitor and enzyme preceding covalent bond formation. The model is shown in eq 1, where E·I is the reversible complex, E–I the inactive enzyme, K_I the steady-state constant of inactivation, V_{sat} the maximum rate of inactivation obtained at saturation, and k_2 the first-order rate constant. The irreversibility of the second step in eq 1 was proven by the inability of

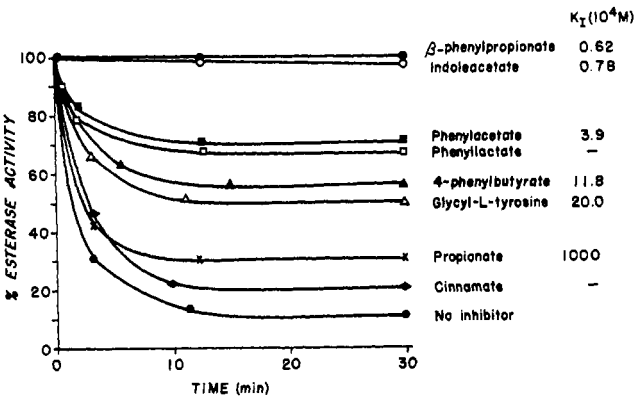

FIGURE 2: Rate of inactivation of carboxypeptidase A (Anson) (1.8×10^{-5} M) by reagent K (1.89×10^{-3} M) in 1 M NaCl–0.005 M potassium phosphate (pH 6.4) at 25°, in the presence of 2×10^{-3} M of various inhibitors. Esterase activity was measured with HPLA. The K_I values were taken from Elkins-Kaufman and Neurath (1949) and Smith *et al.* (1951).

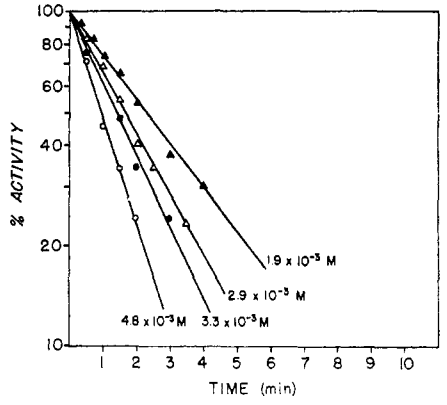

FIGURE 3: Rate of inactivation of carboxypeptidase A (Anson) (1.8×10^{-5} M) as a function of reagent K concentration in 1 M NaCl–0.005 M potassium phosphate (pH 6.4) at 25°. Esterase activity was measured with HPLA. The pseudo-first-order rate constants, k_{app}, were calculated from the slopes according to eq 7 and 8 (see text and Table I). Small errors in pH as well as different batches of reagent K were found to affect the value of these constants.

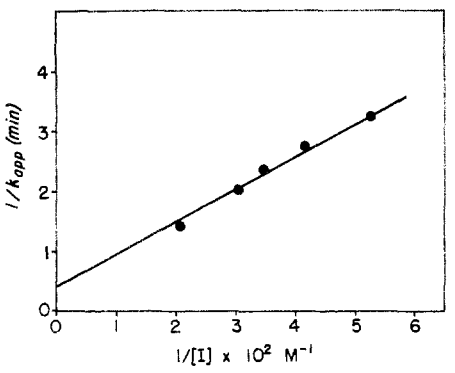

FIGURE 4: Dependence of the reciprocal of the pseudo-first-order rate constants (calculated from Figure 3) on the reciprocal reagent K concentration as defined by eq 9 (see text). k_2 and K_I were calculated from the y intercept and the slope, respectively, using the east-squares method.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_2} E\text{–}I \tag{1}$$

$$K_I = \frac{[E][I]}{[E\cdot I]} \tag{2}$$

$$V_{sat} = k_2[E\cdot I] \tag{3}$$

K-CPA solutions to regain activity at 25° after more than 12 hr. The rate of inhibition was measured by removing small aliquots (20 μl) from the reaction mixture at prescribed times and immediately adding them to assay solutions (3 ml). k_2 and K_I were calculated by plotting the reciprocal of the pseudo-first-order rate constants *vs.* the reciprocal of the reagent K concentrations as shown in eq 9 (Kitz and Wilson, 1962). The following relationships apply

$$[E_0] = [E'] + [E\text{–}I] \tag{4}$$

$$[E'] = [E] + [E\cdot I] \tag{5}$$

where $[E_0]$ is the total enzyme concentration and [E] the concentration of unbound enzyme. Under nonsaturation conditions, the rate equation is

$$v = -\frac{d[E']}{dt} = k_2\left(\frac{[E']}{\frac{K_I}{[I]} + 1}\right) \tag{6}$$

Integrating

$$\ln\frac{[E']}{[E_0]} = -\frac{k_2 t}{\frac{K_I}{[I]} + 1} \tag{7}$$

the rate of inhibition can be obtained by plotting the log of per cent activity remaining *vs.* time. The results are shown in Figure 3 and Table I. As predicted, the inhibition is pseudo first order in enzyme concentration in all cases. Therefore, substituting in eq 7

$$k_{app} = \frac{k_2}{\frac{K_I}{[I]} + 1} \tag{8}$$

and rearranging (Kitz and Wilson, 1962)

$$\frac{1}{k_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \tag{9}$$

Saturation kinetics resulting from the formation of an [enzyme·reagent] reversible complex were demonstrated by plotting data according to eq 9 (Figure 4).

An apparent binding constant, $K_{I,app} = 3.77 \pm 0.1 \times 10^{-2}$ M, and a first-order rate constant at saturation, $k_2 = 2.19 \pm 0.26$ min^{-1} at pH 6.4, were calculated from the slope and intercept of the linear plot, respectively. The pH dependence of the pseudo-first-order rate constant of inhibition, k_{app}, is shown in Table I and Figure 5. The solid line represents a theoretical curve calculated from eq 10

$$k_{app} = \frac{k'_{app}}{1 + \frac{[H^+]}{K_a}} \tag{10}$$

assuming one ionizing group of $pK_a = 7.0$ and $k'_{app} = 1.81$ min^{-1}. k'_{app} is the pH-dependent first-order rate constant obtained experimentally above pH 8. This was the highest rate constant obtained in the basic pH range under these conditions (see legend to Figure 5).

Characterization of the Chemically Modified Enzyme. The incorporation of reagent K into the enzyme was estimated by measuring the ethylamine content of samples at prescribed reaction times. The incorporation of reagent was proportional to the loss of enzymatic activity up to about 50% inactivation, as shown in Figure 6. Deviation from linearity beyond 50% inactivation is indicative of modification at other sites. Extrapolation to 100% inactivation indicates that the loss of activity results from the incorporation of 2 moles of reagent/mole of enzyme. However, when the reaction was carried out in the presence of 2×10^{-3} M β-phenylpropionate to protect the enzyme (Figure 1), the reagent was nevertheless incorporated, as demonstrated in Figure 7. After 20 min, when the reaction was essentially completed, the difference between the enzyme modified in the presence and absence of β-phenylpropionate, respectively, was 1 mole/mole of protein.[5] The

[5] The data in Figure 7 show a difference of about 0.7 residue after 20-min reaction and 14% active enzyme still remaining. When higher concentrations of reagent were used, less active enzyme remained after 20 min and the difference of bound reagent approached 1 mole.

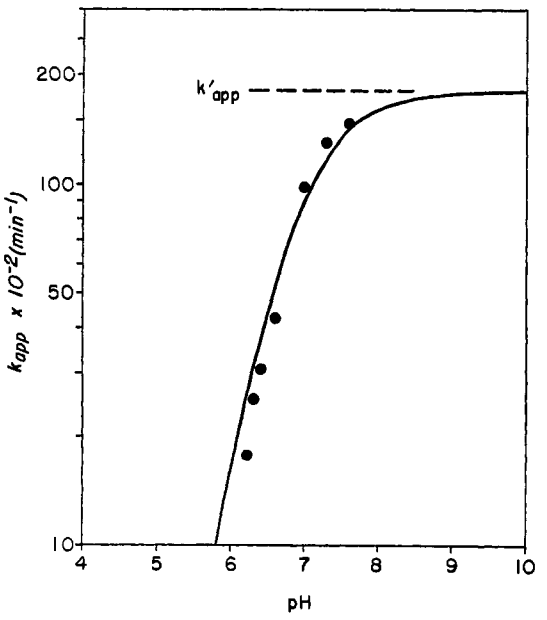

FIGURE 5: Dependence of pH on the pseudo-first-order rate constant of inhibition of carboxypeptidase A (Anson) by reagent K in 1 M NaCl–0.005 M potassium phosphate at 25°. Enzyme concentration was 1.85×10^{-5} M. k_{app} values were taken from Table I. The solid line is a theoretical curve calculated from eq 10, assuming a single ionizing group of $pK_a = 7.0$ and $k'_{app} = 1.81$ min^{-1}. k'_{app} was experimentally determined and was the highest value obtained in the basic range. k'_{app} could not be included in the graph because of very large fluctuations in pH during its determination.

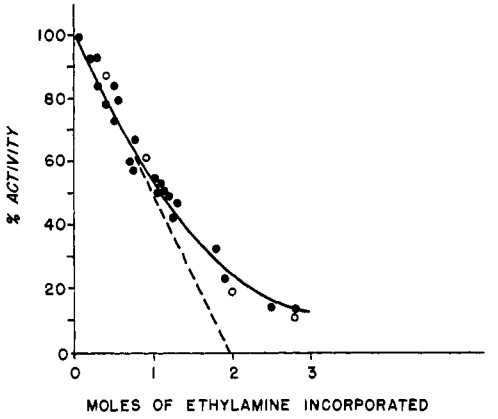

FIGURE 6: Effect of the degree of reagent K incorporation on the enzymatic activity. Same experimental conditions as described in Figure 1. (●) Esterase activity; (○) peptidase activity.

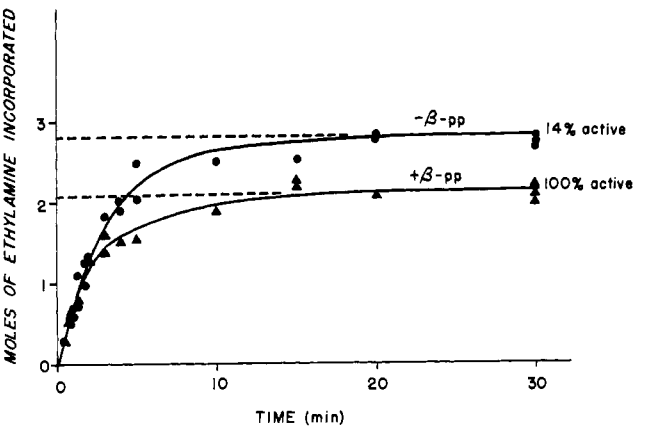

FIGURE 7: Rate of incorporation of reagent in the absence and presence of β-phenylpropionate. Same experimental conditions as described in Figure 1. (●) The absence of β-phenylpropionate; (▲) the presence of β-phenylpropionate.

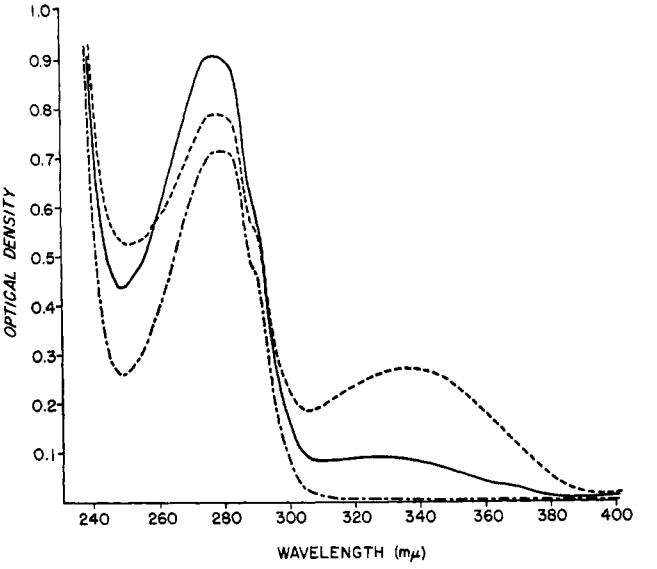

FIGURE 8: Absorption spectra in 1 M NaCl–0.005 M potassium phosphate, pH 6.4 at 25°. (–·–·–) indicates native CPAγ^{Val}; (– – – –) K-CPAγ^{Val} prepared in expt 6 (Table II), ϵ_{278} 8.43×10^4 M^{-1} cm^{-1}; (———) M-CPAγ^{Val} prepared in expt 7 (Table II), ϵ_{278} 7.8×10^4 M^{-1} cm^{-1}.

data shown in Figures 6 and 7 can then be best interpreted by assuming that more than one site is modified at the same time, and that in the presence of β-phenylpropionate, the incorporation at the nonfunctional sites is faster than in the absence of inhibitor. After correcting for the amount of reagent bound at these nonfunctional sites, the per cent loss of activity was proportional to about 1 mole of reagent/mole of enzyme, substantiating the data shown in Figure 7. The enzyme was also inactivated in the presence of *trans*-cinnamate (Figure 2), resulting in the incorporation of 2.8 moles of ethylamine/mole of enzyme after 30-min reaction time.

The ultraviolet absorption properties of the modified enzyme are shown in Figure 8 and Table II. Chemical modification results in the appearance of a broad absorption peak with a maximum at 340 nm and an increase in absorption in the region of 260–270 nm (Figure 8) where enol esters absorb (α-acetoxy-*N*-methylcinnamide[6] in CH_2Cl_2, λ_{max} 267 nm (ϵ 18,700), Woodward and Olofson, 1966). The intensity of absorption of K-CPA at these two regions of the ultraviolet spectrum can serve as a qualitative measure of the degree of chemical modification and can be expressed as the ratios OD 280:250 (Bodlaender *et al.*, 1969) and OD 280:340. The first ratio decreases from 2.75, the value for the native enzyme, to about 1.5 and 1.0 when approximately 1 and 2 moles of reagent, respectively, are incorporated per mole of enzyme (expt 1, 2, and 6, Table II). The second ratio decreases from ∞, for the native enzyme after correcting for scattering, to

[6] Obtained from the reaction of *N*-methyl-5-phenylisoxazolium fluoroborate with sodium acetate.

TABLE II: Characterization of Derivatives of Modified Carboxypeptidase A.

	K-CPA						β-PP-K-CPA[a]			M-CPA							
		Ethylamine (Moles/Mole of Protein)						Ethylamine (Moles/Mole)			Ethylamine (Moles/Mole)			D[e]		E[f]	
Expt	Act. (%)	A[b]	B[c]	C[d]	OD 280:250	OD 280:340	Act. (%)	A	C	Act. (%)	B	% Loss	^{14}C Incorp (Mole/Mole)	OD 280:250	OD 280:340	OD 280:250	OD 280:340
1	11	2.82	2.07		0.89	2.44											
2[g]	16	2.50	1.56		1.29	2.36				9	0.84	46		1.92	6.75		
3[g]	15				1.18	2.40				14	0.79			1.75	5.78	1.74	5.21
4[g]	40	1.58	1.35		1.36	3.74				25	0.86	46		1.99	7.97		
5[h]	41	1.49	1.25								0.51	50	0.74			2.0	10.4
6[g]	51	1.2	1.0		1.49	2.85				34	0.43	57		1.92	8.01	1.94	7.57
7[h]	56	1.2								35						2.1	10.2
8[h]	57	1.1								30	0.3	73				2.4	12.7
9[h]	43	1.44									0.3	79				2.2	42.0
10[h]	34	1.33									0.45	66					
11[h]	46	1.0									0.1	90					
12[i]	42	1.51		1.50							0.3	80					
13[h]							100	1.0		80	0.14	86	0.80			2.5	12.7
14							100	2.2									
15[i]							100	1.8	1.4		0.43	70	1.3				
16[i]	19												2.2				

[a] Denotes K-CPA prepared in the presence of β-phenylpropionate (see text). [b] Ethylamine determinations were performed immediately after stopping the reaction with either 98% formic acid or 13.3 M sodium formate. [c] Ethylamine determinations were performed 12 hr after stopping the reaction. Protein solution was gel filtered and concentrated at 4°. [d] Ethylamine determinations were performed 8 hr after stopping the reaction. Protein solution was concentrated at 4° and precipitated with 98% formic acid. [e] Nucleophilic displacement was performed according to method 1 (see Experimental Section). [f] Nucleophilic displacement was performed according to method 2 (see Experimental Section). [g] Nucleophilic displacement was performed at 25° for 15 hr. [h] Nucleophilic displacement was performed at 25° for 36 hr. [i] Nucleophilic displacement was performed at 37° for 15 hr.

about 3 and 2.5 when 1 and 2 moles of reagent, respectively, are incorporated. Although these ratios are useful in estimating the degree of modification, they cannot be used for the quantitative determinations of bound reagent because of the relatively slower formation of an imide isomer resulting from a base-catalyzed rearrangement of the enol ester (Figure 9, step VI, Woodward and Olofson, 1966; Bodlaender *et al.*, 1969). This new product has different absorption properties (*N*-acetyl-*N*-methylbenzoylacetamide[7] in ethanol, λ_{max} 242 nm (ε 12,300), Woodward and Olofson, 1966) resulting in lower OD 280:250 ratios. Although the formation of this product could not be demonstrated spectrally in the 250-nm region due to some loss of bound reagent, which would result in an increase of the 280:250 OD ratio, it was detected in the near-uv region when a solution of K-CPA was allowed to react for 16 hr at 25° in 0.1 M Tris-Cl (pH 7.2). The spectrum in the 340-nm region underwent a blue shift with a new λ_{max} at 325 nm. This spectral change also took place during nucleophilic displacement with methoxamine (Figure 8) and is indicative of the formation of a new derivative, possibly the imide product discussed above.

The amino acid compositions of the modified enzymes prepared in the presence and absence of β-phenylpropionate could not be distinguished from that of the native enzyme. Lysine, histidine, arginine, and tyrosine, the residues most likely to be modified other than carboxyl groups, exhibited identical values in all cases irrespective of the degree of modification.

As expected from the results just discussed, it was found that several derivatives of K-CPA were produced during the chemical modification. However, attempts to purify these derivatives have not met with success. The reaction of CPA_{γ}^{Val}, a chromatographically pure species of the enzyme (Pétra and Neurath, 1969), with reagent K produced a complex mixture when chromatographed on DE-52 and could not be satisfactorily separated into pure fractions. This did not interfere, however, with the final interpretation of the results, and subsequent experiments were carried out on the mixture of derivatives.

Stability of the Enol Ester Bonds in the Modified Enzyme. The stability of the label introduced during modification was tested by determining the ethylamine content of K-CPA solutions which had been left at pH 6.4 and 0–4° for 12 hr. Little label was lost under these conditions from modified enzyme originally containing 1–1.5 moles of reagent when subjected to gel filtration on Sephadex G-25 (expt 4, 5, and 6, Table II) or concentrated in the Diaflo and precipitated with 98% formic acid (expt 12, Table II). Furthermore, when the same solution was left at room temperature (23–25°) for 16 hr at pH 6.4, the

[7] Obtained from the reaction of *N*-acetyl-*N*-methylbenzoylacetamide with ethanol.

spectral properties remained unchanged (in contrast to the reaction in 0.1 M Tris-Cl, pH 7.2) and no label was lost. However, a significant amount of bound reagent was lost from the protein originally containing more than 2 moles of reagent (expt 1 and 2, Table II). Since no enzymatic activity was regenerated during this process, the reagent was probably located on unusually reactive, nonfunctional, carboxyl groups, creating unstable enol ester bonds. No further loss of label could be demonstrated when the ethylamine content approached 2 moles of reagent/mole of enzyme.

Nucleophilic Displacement of the Bound Reagent with Methoxamine. PREPARATION OF METHOXAMIDE CPA$_\gamma^{Val}$. The modified enzymes prepared in the presence and absence of β-phenylpropionate were reacted with methoxamine in an attempt to displace the label by nucleophilic attack at the carbonyl group of the enol ester to form an amide (Figure 9, step IV). Such reactions would not only serve to provide strong evidence for the modification of carboxylic acid side chains of the protein but would also introduce a more stable label required for the location of the modified residues in the primary sequence of the enzyme (Pétra and Neurath, 1971). Depending on the conditions of methoxamine treatment, loss of ethylamine ranged from 50 to 90% (Table II). In the presence of [^{14}C]-methoxamine the radioactive label was incorporated into the protein with concomitant loss of ethylamine (expt 5, 13, 15, and 16, Table II), resulting in the formation of a new derivative, methoxamide CPA$_\gamma^{Val}$ (M-CPA). In some cases the amount of incorporation measured by the ethylamine content was not quantitatively correlated with the incorporation of radioactivity. Since the errors involved in the determination of low amounts of ethylamine are relatively large, the radioactivity data are a more accurate measure of methoxamine incorporation. As predicted from Figure 7, expt 15 and 16 (Table II) show that the difference of incorporation in the presence and absence of β-phenylpropionate is 0.9 mole of reagent/mole of protein. The relatively high enzymatic activity of some samples of M-CPA (expt 4, 6, 7, and 8, Table II) was due to the presence of residual active enzyme in the initial inhibition reaction (terminated at 40–50% inhibition) which could not be removed chromatographically. M-CPA derived from 100% active modified enzyme, prepared in the presence of β-phenylpropionate, was 80% active (expt 13, Table II). The 20–40% loss of activity during nucleophilic displacement was confirmed by control experiments with native enzyme.

The absorption properties of M-CPA are shown in Figure 8 and Table II. In every case, both OD ratios, 280:250 and 280:340, increased with a corresponding decrease in ethylamine content (expt 2, 3, 4, and 6, Table II). When the displacement reaction was continued for longer periods of time, significant amounts of ethylamine remained bound to the enzyme and could not be removed. In addition, a blue shift in the 340-nm region was apparent in the ultraviolet spectrum of all M-CPA samples. These results strongly suggest the accumulation of a new derivative as reaction proceeded, and the amount of bound ethylamine remaining can be used as a measure of its formation. This competing reaction is most likely the rearrangement of the enol ester to the corresponding imide previously discussed (Figure 9, step VI), the latter being a much weaker acylating agent than the former (Bodlaender *et al.*, 1969).

The presence of degradation products of excess Woodward's reagent K did not interfere with the nucleophilic displacement; the OD ratios were identical within experimental error in both cases (expt 3 and 6, columns D and E, Table II).

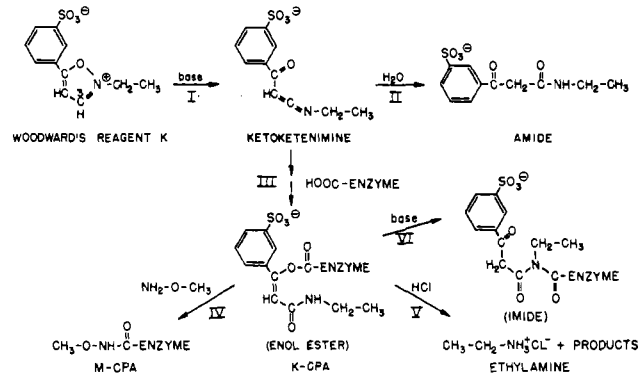

FIGURE 9: Scheme relating the various steps involved in the chemical modification of carboxypeptidase A with Woodward's reagent K.

Discussion

The use of isoxazolium salts for the activation of carboxyl groups in nonaqueous media has been described in detail by Woodward and coworkers (Woodward and Olofson, 1966; Woodward *et al.*, 1966). These authors were mainly interested in characterizing new reagents for the synthesis of peptides. Recently, the studies were extended to the modification of carboxylic acid side chains of proteins, trypsin in particular, in aqueous solutions (Bodlaender *et al.*, 1969; Feinstein *et al.*, 1969). The different steps involved in the overall reaction are schematically represented in Figure 9. The reaction proceeds by the irreversible formation of a ketoketenimine (step I), structurally related to a carbodiimide, which in turn reacts with a carboxyl group to give an enol ester (step III). The incorporation of reagent can be measured by the amount of ethylamine released after acid hydrolysis of the derivatized protein (step V). Finally, the reagent can be displaced from the enzyme with an appropriate nucleophile to yield a more stable intermediate (step IV).

There are two important side reactions which will decrease the yield of methoxamine incorporation, *i.e.*, the rapid hydrolysis of the ketoketenimine above pH 3 to form the corresponding amide (step II), and the base-catalyzed rearrangement of the enol ester to the imide (step VI). Even though the former could not be controlled since the reaction is carried out in an aqueous medium, its effect on the overall production of enol ester was overcome by using higher initial concentrations of reagent to yield pseudo-first-order kinetics of inhibition. The latter, however, could not be prevented because its rate of formation was sufficiently high to compete with the rate of nucleophilic displacement by methoxamine. Since the imide is a much weaker acylating agent than the enol ester, its accumulation during the reaction can be measured by estimating the amount of bound reagent remaining at the end of the nucleophilic displacement (Bodlaender *et al.*, 1969). Values ranging from 20 to 50% rearrangement to the imide were obtained in this study.

The presence of a carboxyl group in the active site of carboxypeptidase A and its participation in the mechanism of action are strongly supported by the nature of the data presented in this paper and by analogous experiments employing a carbodiimide by Riordan and Hayashida (1970) which appeared in press while this manuscript was in preparation. Peptidase and esterase activities are simultaneously abolished and the rapid inactivation of the enzyme is proportional to the incorporation of about 1 mole of reagent/mole of enzyme. Protection against inactivation by β-phenylpropionate as

well as other reversible inhibitors was actually the first indication that the reagent was binding in the active center of the enzyme.

Kinetic studies of the inhibition process revealed the existence of an [enzyme·reagent] complex with a steady-state constant, $K_{I,app}$, of $3.77 \pm 0.1 \times 10^{-2}$ M at pH 6.4. Assuming that this constant does not vary greatly between pH 6.4 and 7.5, the value is 10–100 times greater than the previously reported K_I values for most inhibitors which compete effectively for peptide substrates at pH 7.5 (Figure 2, Auld and Vallee, 1970a). The reagent is also bound poorly in comparison to *N*-bromoacetyl-*N*-methyl-L-phenylalanine, a recently described affinity label for carboxypeptidase A (Hass and Neurath, 1971). The high value of $K_{I,app}$ is not surprising considering that the overall structure of reagent K is not entirely analogous to that of most carboxypeptidase A substrates. The large sulfonic acid group, although providing a negative charge, would be expected to induce perturbation in the environment of the active center. Furthermore, the nature of the binding process may be complicated by the reaction involving the conversion of reagent K to the ketoketenimine (step I, Figure 9). In fact, both of these compounds may exhibit different affinities for the active site, thereby introducing difficulty in the physical interpretation of $K_{I,app}$. Although a rate constant for the conversion of reagent K into the ketoketenimine in aqueous solution at pH 6.4 has not been reported, indications are that it is quite large. This problem brings to mind another, *i.e.*, does the protein have any influence on the conversion of reagent K to the ketoketenimine? Woodward and coworkers have shown that the mechanism of the reaction involves the formation of an ylide produced by the abstraction of the proton at position 3 in reagent K (Figure 9). This reaction takes place in the presence of a base such as OH^- in aqueous solution. However, it has been suggested that upon interaction with a protein, a carboxylic acid side chain could serve as a base to promote opening of the ring and form the ketoketenimine which subsequently would react with another carboxyl group to form the enol ester (Feinstein *et al.*, 1969). In the case of carboxypeptidase A, the data presented cannot rule out this possibility.

The inactivation of the enzyme reagent K is dependent on pH with an extrapolated intercept corresponding to $pK_a \sim 7$ (Figure 5). Since this determination was performed at reagent K concentrations (1.9×10^{-3} to 4.8×10^{-3} M) far removed from saturation conditions, it is not possible *a priori* to relate this pK to a single functional group of the enzyme. However, the pH dependence of inhibition by reagent K is almost identical to that of the enzyme-catalyzed hydrolysis of peptide and ester substrates in the acid region (Neurath and Schwert, 1950; Riordan and Vallee, 1963; Carson and Kaiser, 1966; Auld and Vallee, 1970b). Carson and Kaiser (1966) have shown that the carboxypeptidase A catalyzed hydrolysis of *O*-acetyl-L-mandelate in the acid pH range is dependent on the ionization of a group on the free enzyme having a pK_a of 6.9. Under saturation conditions these workers obtained a pK_a value of 7.2, indicating a slight perturbation of this group when the substrate was bound. Even though the structure of this ester substrate is quite different from that of reagent K, there is a remarkable similarity between the pK_a values obtained in both studies. This agreement strongly suggests that the same group which is inactivated by reagent K is essential to the mechanism of action. These findings correlate well with the pH dependence of inhibition of carboxypeptidase A_γ^{Leu} by *N*-bromoacetyl-*N*-methyl-L-phenylalanine (Hass and Neurath, 1971).

The nucleophilic displacement of the label by methoxamine results in the formation of stable derivatives. These experiments not only provide further evidence for modification at carboxyl groups but also have made possible additional studies leading to the identification of the modified sites in the primary structure of the enzyme. These results are reported in the accompanying paper (Pétra and Neurath, 1971).

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