MODIFICATION OF AMINO ACID RESIDUES IN THE S₁' BINDING SITE OF CARBOXYPEPTIDASE Y

by

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Carboxypeptidase Y was treated under different conditions with various reagents in order to chemically modify amino acid side-chains located in the S₁' binding site. Treatment of carboxypeptidase Y with H₂O₂ in phosphate buffer caused a reduction in the peptidase activity towards FA-Phe¹-Leu-OH to 25 % of the control while the esterase activity towards FA-Phe²-OMe, the amidase activity towards FA-Phe²-NH₂ and the peptidyl amino acid amide hydrolase activity towards FA-Phe²-Gly-NH₂ were much less affected. The loss of peptidase activity could be correlated with the oxidation of a single methionyl residue, thus forming a methionyl sulfoxide derivative. When the reaction was performed in acetate buffer under otherwise identical conditions an additional methionyl residue was oxidized with the result that all activities of the enzyme increased while the heat stability of the enzyme decreased drastically.

¹⁴C-Fluorodinitrobenzene influenced the activities of carboxypeptidase Y in a manner similar to that observed with H₂O₂ in phosphate buffer. The loss of peptidase activity could be correlated with the incorporation of a single equivalent of reagent as an O-DNP-tyrosyl derivative.

The carboxypeptidase Y derivatives containing a single methionyl sulfoxide residue or an O-DNP-tyrosyl residue both exhibited altered specificity with respect to the P₁' position of the substrate. These results indicate that both the methionyl residue and the tyrosyl residue are located in the S₁' binding site.

1. INTRODUCTION

In a recent paper from this laboratory it was demonstrated that the S₁' binding site of CPD-Y contains a methionyl residue which could be modified with alkylating reagents, e.g. iodoace-

tamide or phenacylbromide (7), thus in both cases introducing a sulfonium ion and a rather bulky group. These derivatives of CPD-Y exhibit specificities with respect to the P₁' position of all types of substrates which drastically deviate

Abbreviations: Bz = benzoyl; Bzl = benzyl; CPD-Y = carboxypeptidase Y; DNP = dinitrophenyl; EDTA = ethylenediamine tetraacetic acid, sodium salt; FA = furylacryloyl; Hepes = N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; IAA-CPD-Y = CPD-Y modified with iodoacetamide; Mes = 2-(N-morpholino)ethane sulfonic acid; Met-SO = methionine sulfoxide; Met-SO-CPD-Y = CPD-Y containing one methionyl sulfoxide residue; (Met-SO)₂-CPD-Y = CPD-Y containing two methionyl sulfoxide residues; m-NPAB-CPD-Y = CPD-Y modified with meta-nitrophenacylbromide; PAB-CPD-Y = CPD-Y modified with phenacylbromide; p-HMB = para-hydroxymercuribenzoate; p-NPAB-CPD-Y = CPD-Y modified with para-nitrophenacylbromide. The binding site notation is that of SCHECHTER and BERGER (17). Accordingly, the binding site for the C-terminal amino acid residue of the substrate is denoted S₁' and those for the amino acid residues in the amino terminal direction away from the scissile bond is denoted S₁, S₂,..., S_n.

from that of unmodified CPD-Y (7). Other amino acid side-chains might be located in the S'_1 binding site and hence, it was of interest to establish whether the introduction of other groups in this portion of the enzyme would cause similar drastic changes in its specificity. The present paper describes the oxidation of the methionyl in the S'_1 binding site and in addition, the modification of a tyrosyl residue situated in the same area. It is demonstrated that the specificities of these modified enzymes deviate less from that of unmodified CPD-Y than observed for the derivatives containing the methionine sulfonium groups (7). It is further shown that the modification of another methionyl residue located in a different portion of the enzyme molecule also changes the catalytic properties of the enzyme.

2. MATERIALS AND METHODS

2.1. Materials

The following compounds were synthesized as previously described: FA-L-Phe-NH₂ (4), FA-L-Phe-L-Gly-NH₂ (4), FA-L-Phe-L-Gly-OH (4), FA-L-Phe-L-Ala-OH (7), FA-L-Phe-L-Leu-OH (7), FA-L-Ala-OBzl (7), FA-L-Ala-OEt (7), FA-L-Phe-L-Val-NH₂ (7), CABS-Sephadex (4). Bio-gel P-4 was obtained from Biorad, USA, CPD-Y from Carlsberg Biotechnology Ltd., Tagensvej 16, DK-2200 Copenhagen N; FA-L-Phe-OMe and Z-D-Ala-L-Phe-OH from Bachem, Switzerland; (D,L)-benzylsuccinic acid from Burdick and Jackson, USA; N-bromosuccinimide, acrylonitrile and phenacylbromide from Fluka, Switzerland; Mes, Hepes, β -propiolactone, iodoacetic acid and FDNB from Sigma, USA; trinitrobenzene sulfonic acid from Pierce, USA; ICI and tetranitromethane from EGA Chemie, W. Germany; ¹⁴C-FDNB from Amersham, England. All other chemicals and solvents were of analytical purity and obtained from Merck, W. Germany.

2.2. Methods

2.2.1. Modifications of CPD-Y

The influence of various reagents on the esterase, amidase, peptidyl amino acid amide hydrolase and peptidase activities of CPD-Y was

studied using FA-Phe[↓]OMe, FA-Phe[↓]NH₂, FA-Phe[↓]Gly-NH₂ and FA-Phe[↓]Leu-OH as substrates, respectively. The assay with FA-Phe[↓]Leu-OH was performed in 0.05 M-Mes, 1 mM-EDTA, pH 6.5, and with the other substrates 0.05 M-Hepes, 1 mM-EDTA, pH 7.5 was used. The assay mixture consisted of 965 μ l buffer, 25 μ l 12 mM substrate dissolved in methanol and 10 μ l aliquots of the reaction mixture, added directly or after an appropriate dilution in water. The rate of hydrolysis was followed spectrophotometrically at 337 nm using a Cary 219 spectrophotometer, thermostated at 25 °C. All modification reactions of CPD-Y were carried out at room temperature using an enzyme concentration of 5.3 mg/ml. Control reactions without reagent added ensured that the enzyme was stable under the employed conditions. Reactions were terminated by separating the enzyme from reagent by gel filtration on Biogel P-4, equilibrated with water. The bed volume was 20-30 times the volume of the sample applied. The modified preparations of CPD-Y were stored frozen in water at -18 °C. Modification of CPD-Y in the presence of inhibitor was performed by addition of 50 μ l 1 M-Z-D-Ala-L-Phe-OH or 12.5 μ l 0.4 M-(D,L)-benzylsuccinic acid dissolved in ethanol per ml reaction medium, followed by readjustment of pH.

2.2.2. Characterization of modified CPD-Y

The preparations of modified enzyme obtained after gel filtration were analyzed for amino acid composition and for the incorporation of radioactively labelled reagent (only for FDNB). The amino acid composition was determined after acid hydrolysis for 24 hours in vacuo using a Durrum D 500 amino acid analyzer. The content of Tyr was determined employing a special program capable of separating it from glucosamine, another component of CPD-Y. Methionine sulfoxide was determined indirectly since it reverts almost completely to methionine during acid hydrolysis (15). The following procedure converts all methionine in unmodified CPD-Y to homoserine and homoserine lactone: 1 mg lyophilized enzyme was dissolved in 140 μ l 70% formic acid and the solution was purged with nitrogen for 10 min. Cyanogen bromide (10 mg) was added and the solution again purged

with nitrogen for 1 min. The reaction vessel was sealed and stored in the dark for 22 hours. Nitrogen was then bubbled through the solution for 3 hours, the sample evaporated to dryness, subjected to acid hydrolysis and amino acid analysis. Since methionine sulfoxide is resistant to cyanogen bromide treatment (15) its content could be quantitated as methionine in the acid hydrolysates of cyanogen bromide treated enzyme preparations.

Spectra were recorded using a Cary 219 spectrophotometer, the content of free cysteine was estimated by titration with para-hydroxymercuribenzoate as described by RIORDAN and VALLEE (14) and the carbohydrate content was estimated by the phenol-sulfuric acid method of DUBOIS (8). ^{14}C -measurements were performed by liquid scintillation using a Beckman scintillation counter, Model LS 3155 T. The stoichiometry of the incorporated reagent was based on spectrophotometrically determined enzyme concentrations using $A_{280} = 14.8$ (10).

Modification of Met-SO-CPD-Y with phenacylbromide was performed at an enzyme concentration of 5.0 mg/ml in 0.03 M- NaH_2PO_4 , 0.12 M- NaCl , 2.5% methanol, 6.2 mM-phenacylbromide, pH 6.5 as previously described (7).

2.2.3. Determination of kinetic constants

Modified CPD-Y was separated from unmodified enzyme by affinity chromatography as previously described (7) before it was characterized kinetically. The kinetic constants for the hydrolysis of various FA-peptide and ester substrates were determined from Lineweaver-Burk plots. Such determinations were not possible with peptide amides due to their low solubility combined with high K_m values. With these substrates only k_{cat}/K_m was determined by following the hydrolysis to completion at a single concentration of substrate and using the integrated form of the Michaelis-Menten equation as described in ref. 18. With FA-Phe-Gly- NH_2 and FA-Phe-Val- NH_2 direct amino acid analysis of the assay mixture ascertained that the enzyme only released H-Gly- NH_2 and H-Val- NH_2 , respectively, and not NH_3 followed by the C-terminal amino acid. Hydrolysis rates were determined spectrophotometrically at 329 – 358 nm

and 25 °C. Peptide substrates were assayed in 0.05 M-Mes, 1 mM-EDTA, pH 6.5 whereas ester and amide substrates were assayed in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5.

2.2.4. Stability of CPD-Y derivatives

The stability of CPD-Y derivatives was investigated by incubating the enzymes at different temperatures in 0.05 M-Mes, 1 mM-EDTA, pH 6.0 at a concentration of 0.1 – 0.2 mg/ml. The inactivation was followed by assay of the activity towards FA-Phe $^{\downarrow}$ Leu-OH. The influence of the temperature on the rate constant k was determined and the activation energy E_a was determined from the Arrhenius equation, $k = A \cdot e^{-(E_a/RT)}$, by plotting $\log k$ as a function of T^{-1} .

3. RESULTS

3.1. Selection of reagents

Since CPD-Y exhibits a preference for hydrophobic groups in the P_1' position of all types of substrates (9) it is probable that the S_1' binding site contains mostly hydrophobic amino acid residues. Among these Tyr, Trp, Cys and Met contain functional groups which may be modified, and CPD-Y was consequently treated with a series of reagents known to attack these amino acid side-chains (Table I). During the reactions the activities of the enzyme towards FA-Phe $^{\downarrow}$ Leu-OH and FA-Phe $^{\downarrow}$ OMe were assayed since previous studies (7) had shown that the introduction of labels in the S_1' binding site of the enzyme would reduce the activity of the enzyme towards FA-Phe $^{\downarrow}$ Leu-OH which contains the bulky -Leu-OH group at the P_1' position to a larger extent than the activity towards FA-Phe $^{\downarrow}$ OMe which contains the small -OMe group in this position. It is observed that some of the reagents abolished both activities completely while others had no influence on either activity and both these types of reagents are without interest for the present study (Table I). However, in the reactions with FDNB and H_2O_2 the activity towards FA-Phe $^{\downarrow}$ Leu-OH was reduced much more than the activity towards FA-Phe $^{\downarrow}$ OMe (Table I) and hence, these two reactions were studied in further detail.

Table 1.
Chemical modifications of carboxypeptidase Y

Reagent	% Residual activity		
	FA-Phe-Leu-OH	FA-Phe-OMe	
Trinitrobenzene-sulfonic acid	100	100	a)
Fluorodinitrobenzene	5	100	b)
Ethyleneimine	100	100	c)
Acrylonitrile	100	100	d)
Methyl iodide	45	45	e)
β -propiolactone	2	5	f)
Iodoacetate	100	100	g)
ICl	0	0	h)
Iodine	0	0	i)
Tetranitromethane	25	40	j)
H ₂ O ₂	27	88	k)
N-bromosuccinimide	15	30	l)

The reactions were performed at an enzyme concentration of 4–6 mg/ml under the following conditions: a) 0.1 M-NaH₂PO₄, 11 mM reagent, pH 8.0, reaction time: 210 min., b) 0.1 M-NaH₂PO₄, 14 mM reagent, pH 8.0, reaction time: 300 min., c) 0.05 M-NaHCO₃, 20 mM reagent, pH 8.6, reaction time: 300 min., d) 0.1 M-NaH₂PO₄, 38 mM reagent, pH 8.0, reaction time: 90 min., e) 0.1 M-NaHCO₃, 100 mM reagent, pH 9.0, reaction time: 300 min., f) in water (pH stat), 600 mM reagent, pH 4.5, reaction time: 75 min., g) 0.05 M-NaH₂PO₄, 0.2 M reagent, pH 6.5, reaction time: 300 min., h) 0.05 M-NaH₂PO₄, 0.2 M-NaCl, 1.6 mM reagent, pH 6.5, reaction time: 3 min., i) 0.1 M-NaHPO₄, 0.1 M-KI, 1 mM reagent, pH 8.0, reaction time: 5 min., j) 0.1 M-Tris, 3.4 mM reagent, pH 8.0, reaction time: 330 min., k) 0.1 M-NaHPO₄, 0.19 M reagent, pH 4.75, reaction time: 140 min., l) 0.05 M-sodium acetate, 2.5 mM reagent, pH 5.0, reaction time: 40 min.

3.2. Modification of CPD-Y with H₂O₂

The influence of H₂O₂ on the activities of CPD-Y was studied using two different reaction media, i.e. sodium phosphate and sodium acetate buffers. Initially, the reaction was carried out in 0.1 M-NaH₂PO₄, pH 4.75 using 0.19 M-H₂O₂ (Figure 1) and within 2½ hours the peptidase activity of CPD-Y towards FA-Phe[↓]-Leu-OH had decreased to approximately 25% of the control (Figure 1A). Smaller reductions were observed in the esterase activity towards FA-Phe[↓]-OMe, the amidase activity towards FA-Phe[↓]-NH₂ and the peptidyl amino acid amide

Figure 1. Modification of CPD-Y with H₂O₂. Reaction conditions: 5.3 mg/ml CPD-Y, 0.1 M-NaH₂PO₄, 0.19 M-H₂O₂, pH 4.75. The activities of the enzyme towards FA-Phe[↓]-Leu-OH (—○—○—), FA-Phe[↓]-NH₂ (—□—□—), FA-Phe[↓]-OMe (—Δ—Δ—) and FA-Phe[↓]-Gly-NH₂ (—×—×—) were assayed during the reaction (Panel A). At different times aliquots of the reaction mixture were desalted and the amino acid composition determined. The content of Met-SO was expressed as residues per mole enzyme (Panel B).

hydrolase activity towards FA-Phe[↓]-Gly-NH₂. The carbohydrate content and amino acid composition of the modified enzyme, as determined after acid hydrolysis, was identical to that of unmodified CPD-Y, and the UV-spectrum and

Figure 2. Inactivation of CPD-Y (—○—○—), Met-SO-CPD-Y (—Δ—Δ—), (Met-SO)₂-CPD-Y (—□—□—) at 50 °C in 0.05 M-Mes, 1 mM-EDTA, pH 6.5.

Table II.**Kinetic parameters for the hydrolysis of ester, peptide and amide substrates by CPD-Y, DNP-CPD-Y and Met-SO-CPD-Y.**

Substrate	Enzyme	Substrate range (mM)	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (min ⁻¹ · mM ⁻¹)
FA-Phe [↓] OMe	CPD-Y		11000	0.39	28000
	Met-SO-CPD-Y	0.1 – 1.0	14200	1.0	14200
	DNP-CPD-Y	0.06 – 0.4	5800	0.21	28000
FA-Phe [↓] OEt	CPD-Y		11000	0.059	190000
	Met-SO-CPD-Y	0.03 – 3.0	14200	0.14	101000
	DNP-CPD-Y	0.07 – 0.4	3200	0.19	17000
FA-Ala [↓] OEt	CPD-Y		4600	1.0	4600
	Met-SO-CPD-Y	0.05 – 5.0	11900	2.9	4100
	DNP-CPD-Y	0.6 – 5.0	3000	2.9	1030
FA-Ala [↓] OBzl	CPD-Y		9100	0.054	170000
	Met-SO-CPD-Y	0.05 – 0.5	10900	0.67	16300
	DNP-CPD-Y	0.05 – 0.5	4400	0.26	17000
FA-Phe [↓] Gly-OH	CPD-Y		5800	5.4	1100
	Met-SO-CPD-Y	0.7 – 5.0	~ 2300	~ 30	80
	DNP-CPD-Y	0.7 – 5.0	~ 1300	~ 12	110
FA-Phe [↓] Ala-OH	CPD-Y		9700	0.14	61000
	Met-SO-CPD-Y	0.15 – 1.5	4900	2.2	2200
	DNP-CPD-Y	0.15 – 1.5	1100	1.5	730
FA-Phe [↓] Leu-OH	CPD-Y		4900	0.021	230000
	Met-SO-CPD-Y	0.05 – 0.5	1600	0.16	10000
	DNP-CPD-Y	0.05 – 0.5	600	0.26	2300
FA-Phe [↓] NH ₂	CPD-Y				89
	Met-SO-CPD-Y	0.5			72
	DNP-CPD-Y	0.5			42
FA-Phe [↓] Gly-NH ₂	CPD-Y				160
	Met-SO-CPD-Y	0.5			72
	DNP-CPD-Y	0.5			26
FA-Phe [↓] Val-NH ₂	CPD-Y				6200
	Met-SO-CPD-Y	0.1			640
	DNP-CPD-Y	0.1			41

The rates of hydrolysis were determined spectrophotometrically at 329 - 358 nm. For ester and amide substrates assays were performed in 0.05 M-Hepes, 1 mM-EDTA, 2.5% methanol, pH 7.5 and for peptide substrates 0.05 M-Mes, 1 mM-EDTA, pH 6.5 was used. In the case of FA-Phe-Val-NH₂ methanol was replaced with dimethylformamide. The data for CPD-Y were from reference 7.

p-HMB titration indicated that neither the tryptophan content nor the free sulphhydryl group was affected. However, the Met-SO content increased from 0 to 1.0 equivalent during the reaction (Figure 1B).

The modified enzyme (Met-SO-CPD-Y) was

separated from unmodified enzyme by affinity chromatography (see section 2.2.3) and after 2 h 20 min the reaction mixture contained only 4-5% unmodified enzyme. Thus, the activity changes observed in Figure 1A correlates with the modification of a single methionyl residue in

CPD-Y. When the reaction was performed in the presence of the inhibitor benzylsuccinic acid the activities of CPD-Y were essentially unaffected by H_2O_2 within three hours suggesting that the methionyl residue modified in the absence of this inhibitor is located in the active site of CPD-Y.

Met-SO-CPD-Y was found to inactivate at 50 °C in an apparent first order reaction with a rate constant k of $4.9 \cdot 10^{-3} \text{ min}^{-1}$ as compared with $3.3 \cdot 10^{-3} \text{ min}^{-1}$ for the unmodified enzyme (Figure 2). The influence of the temperature on k indicated that this reaction had an activation energy E_a of $320 \text{ kJ} \cdot \text{mol}^{-1}$ for the unmodified enzyme and $310 \text{ kJ} \cdot \text{mol}^{-1}$ for Met-SO-CPD-Y. Thus, the introduction of an oxygen atom on a methionyl residue in CPD-Y forming Met-SO-CPD-Y seems to have only a slight adverse effect on the stability of the enzyme.

Met-SO-CPD-Y was kinetically characterized using a series of FA-substrates which were selected such that they primarily reflected the influence of the modification on the leaving group specificity of CPD-Y. The hydrolysis of all the ester substrates listed in Table II was characterized by increased k_{cat} and K_m values relative to unmodified CPD-Y. k_{cat}/K_m decreased for all the ester substrates but interestingly it decreased to approximately 10% for FA-Ala[↓]OBzl containing a bulky leaving group while only to 50-90% for FA-Phe[↓]OMe, FA-Phe[↓]OEt and FA-Ala[↓]OEt containing non-bulky leaving groups. The hydrolysis of the peptide substrates FA-Phe[↓]Gly-OH, FA-Phe[↓]Ala-OH and FA-Phe[↓]Leu-OH by Met-SO-CPD-Y was also characterized by reduced k_{cat}/K_m values but in contrast to the ester substrates this was both due to a reduction in k_{cat} and an increase in K_m . However, with peptide substrates the size of the leaving group influenced the reduction in k_{cat}/K_m less than observed with the ester substrates. The hydrolysis of FA-Phe[↓]NH₂, FA-Phe[↓]Gly-NH₂ and FA-Phe[↓]Val-NH₂ by Met-SO-CPD-Y were characterized by k_{cat}/K_m values which were 80%, 20% and 5% of those obtained with unmodified CPD-Y, respectively, and, as observed with ester substrates, this suggests that the sulfoxide group in Met-SO-CPD-Y adversely influences the interaction between enzyme and substrates with bulky leaving groups.

The results obtained with Met-SO-CPD-Y are consistent with the modified methionyl residue being located in the S₁ binding site. This location was also assigned to the methionyl residue which reacts with alkylating reagents, e.g. iodoacetamide and phenacylbromide, and to establish whether the site of reaction of H_2O_2 and the alkylating reagents is identical, Met-SO-CPD-Y was treated with phenacylbromide under the conditions which previously had been shown to modify a methionyl residue in CPD-Y (see section 2.2.2) causing drastic alterations of its activities (7). It was found that this treatment of Met-SO-CPD-Y had no influence on any of its activities and consequently, it may be inferred that phenacylbromide does not react with Met-SO-CPD-Y because the methionyl residue it reacts with in unmodified CPD-Y had been oxidized in Met-SO-CPD-Y.

Treatment of CPD-Y with H_2O_2 in 0.1 M-sodium acetate under conditions otherwise identical to those employed in Figure 1 altered the course of the reaction (Figure 3A). In the initial phases of the reaction the activities towards FA-Phe[↓]Leu-OH, FA-Phe[↓]OMe, FA-Phe[↓]NH₂ and FA-Phe[↓]Gly-NH₂ all decreased. However, after approximately one hour a second reaction appeared which caused all activities of CPD-Y to increase over the next seven hours. The modified enzyme obtained after 8 hours reactions contained two equivalents of Met-SO while it was otherwise identical to unmodified CPD-Y in terms of amino acid composition, UV-spectrum and sulfhydryl and carbohydrate content. Using affinity chromatography to separate modified from unmodified enzyme it was found that the preparation obtained after 8 hours reaction contained less than 1% residual CPD-Y and consequently, the activity changes observed in Figure 3A can be correlated with the modification of two methionyl residues. Since the activities of CPD-Y within the first hour of reaction were affected in a manner similar to that observed with phosphate buffer as the reaction medium (Figure 1A) and since a simultaneous increase in the Met-SO content to approximately 1 equivalent is observed (Figure 3B) it is probable that the methionyl residue oxidized in phosphate buffer is also oxidized in this early phase of the reaction in acetate buffer. The

Figure 3. Modification of CPD-Y with H_2O_2 . Reaction conditions: 5.3 mg/ml CPD-Y, 0.1 M-sodium acetate, 0.19 M- H_2O_2 , pH 4.75. The activities of the enzyme were assayed during the reaction (Panel A) and the number of Met-SO residues determined (Panel B) as described in Figure 1.

following phase of the reaction (Figure 3B) then represents the slower oxidation of an additional methionyl residue.

The influence of the reaction medium, phosphate or acetate buffer, on the number of methionyl residues being oxidized with H_2O_2 can be explained either by acetate binding to the enzyme thereby exposing an additional methionyl residue or by the potent oxidizing reagent peracetic acid being formed from H_2O_2 and acetic acid. Of these two possibilities the latter is the more probable since a prior 3 hours incubation of buffer and H_2O_2 before enzyme is added

Figure 4. Modification of CPD-Y in the presence of benzy succinic acid. Reaction conditions: 5.3 mg/ml CPD-Y, 0.1 M-sodium acetate, 5 mM-benzy succinic acid, 1.3% ethanol, 0.19 M- H_2O_2 , pH 4.75. The activities of the enzyme were assayed during the reaction (Panel A) and the number of Met-SO residues determined (Panel B) as described in Figure 1.

increased the rate of the reaction significantly.

(Met-SO)₂-CPD-Y was found to inactivate at 50 °C in an apparent first order reaction with a rate constant k of 0.11 min⁻¹ as compared with $4.9 \cdot 10^{-3}$ min⁻¹ for the Met-SO-CPD-Y. The influence of the temperature on k indicated that this reaction had an activation energy E_a of 200 kJ · mol⁻¹ as compared with 310 kJ · mol⁻¹ for Met-SO-CPD-Y. Thus, the introduction of an oxygen atom on the second methionyl residue appears to have a pronounced destabilizing effect on the enzyme.

When the reaction with H_2O_2 in acetate buffer was performed in the presence of benzy succinic acid the activities of CPD-Y towards FA-Phe¹-NH₂, FA-Phe¹-Gly-NH₂ and FA-Phe¹-OMe increased while that towards FA-Phe¹-Leu-OH remained unchanged (Figure 4A). The modified enzyme deviated from the unmodified enzyme only by its content of Met-SO which increased from 0 to 0.7 equivalents after 10 hours reaction (Figure 4B). The absence of an initial phase where all activities of CPD-Y decrease due to oxidation of the first methionyl residue, as observed in the absence of benzy succinic acid

Figure 5. Modification of CPD-Y with FDNB. Reaction conditions: 3.9 mg/ml CPD-Y, 0.08 M-NaH₂PO₄, 14 mM-¹⁴C-FDNB, 2.5% ethanol, pH 6.0. The activities of the enzyme were measured as described in Figure 1 (Panel A). At various times aliquots of the reaction mixture were desalted and the radioactivity and the amino acid composition determined (Panel B). The radioactivity was expressed as mole DNP/mole enzyme (—●—●—●—). The amino acid composition indicated that only the content of tyrosine was affected and the results were expressed as number of tyrosyl residues modified per mole enzyme (—○—○—○—). Thiolytic of the modified enzyme (see Figure 6) caused a reduction in the ¹⁴C-DNP-content (indicated by an arrow).

(Figure 3A), indicates that this particular methionyl residue is not oxidized in the presence of benzy succinic acid. The activity changes seen in Figure 4A are more likely due to oxidation of the second methionyl residue. Thus, regardless of the reaction medium, phosphate (see above) or acetate buffer, benzy succinic acid protects the methionyl residue which otherwise is the one being most susceptible to oxidation. On the other hand, the rate of oxidation of the second methionyl residue is only reduced by approximately 50% by the presence of benzy succinic acid, suggesting that this methionyl residue is

located in a different portion of the molecule than that to which benzy succinic acid binds.

3.3. Modification with fluorodinitrobenzene (FDNB)

Treatment of CPD-Y with FDNB for five hours at pH 8.0 resulted in the modification of approximately five lysyl residues, two histidyl residues and six tyrosyl residues as determined by amino acid analysis of acid hydrolysates of the modified enzyme. The p-HMB titration indicated that the single cysteinyl residue of the enzyme was not affected by the reaction. By lowering the pH of the reaction medium to 6.0 it was achieved that the reaction was limited to a single tyrosyl residue while less than 0.2 lysyl and histidyl residues were modified. The course of the reaction was studied in detail using ¹⁴C-FDNB as the reagent and the activities of CPD-Y towards FA-Phe²-Leu-OH, FA-Phe²-NH₂ and FA-Phe²-Gly-NH₂ decreased while that towards FA-Phe²-OMe remained constant or increased slightly (Figure 5A). Using affinity chromatography to separate modified from unmodified enzyme (see section 2.2.3) it was established that the reaction mixture after 11 hours (Figure 5A) contained only 10% residual CPD-Y and that the modified enzyme exhibited approximately 5% activity towards FA-Phe²-Leu-OH. Thus, the modification of 90% of the enzyme was correlated with the loss of 0.9 residues tyrosine and with the incorporation of 1.2 equivalents of reagent as determined from the radioactivity measurements (Figure 5 B). These results indicate that it is modification of a single tyrosyl residue with FDNB to form a O-DNP-tyrosyl residue which alters the enzymatic properties of the enzyme.

Tyrosine and histidine can be regenerated from O-DNP-tyrosine and N¹m-DNP-histidine by treatment with thiol compounds without affecting N⁶-DNP-lysine and N⁶-DNP-amino acids (19). Thus, provided a tyrosyl residue is the site of reaction with ¹⁴C-FDNB, treatment with mercaptoethanol should release the radioactive label attached to the enzyme. The preparation of CPD-Y obtained after 11 hours reaction with FDNB (see Figure 5A) was treated with 0.14 M-mercaptoethanol at pH 8.0 and the

reaction was followed by recording the spectrum between 300 and 400 nm (Figure 6). Using the molar extinction of 12500 for S-DNP-mercaptoethanol in dimethylformamide (19) the increase in absorption at 341 nm (see insert) corresponds to a loss of 0.65 equivalent DNP from the modified enzyme. However, the radioactivity content of the enzyme after thiolysis for 2 h 20 min and dialysis corresponded to 0.3 equivalent, i.e. a loss of 0.9 equivalent. This indicates that 0.3 equivalent DNP is attached to lysine while the residual 0.9 equivalent is attached to tyrosine or histidine, and this is consistent with the results of the amino acid analysis which indicates that 0.9 tyrosyl residue and less than 0.2 equivalent histidine was modified by FDNB.

When the reaction was performed in the presence of the competitive inhibitor Z-D-Ala-L-Phe-OH (1) the activity of CPD-Y towards FA-Phe²-Leu-OH was reduced by only 10% within 6 hours of reaction with FDNB. Thus, Z-D-Ala-L-Phe-OH protects the enzyme towards reaction with FDNB, suggesting that the tyrosyl residue modified by this reagent in the absence of Z-D-Ala-L-Phe-OH is located in the active site of the enzyme.

DNP-CPD-Y was characterized kinetically using the series of FA-substrates employed in the characterization of Met-SO-CPD-Y. It is apparent from the results in Table II that DNP-CPD-Y hydrolyzed all ester and peptide substrates with reduced k_{cat} values relative to unmodified CPD-Y and, apart from FA-Phe²-OMe, with increased K_m values. k_{cat}/K_m was influenced by the modification in a manner which depends on the size of the leaving group: for the ester substrate FA-Phe²-OMe, characterized by a non-bulky leaving group, k_{cat}/K_m remained unchanged while it was reduced for the ester substrates with more bulky leaving groups, i.e. FA-Phe²-OEt, FA-Ala²-OEt and FA-Ala²-OBzl. DNP-CPD-Y hydrolyzed all peptide and amide substrates with reduced k_{cat}/K_m values relative to CPD-Y but the magnitude of the reduction depended on the size of the leaving group. Thus, k_{cat}/K_m for the hydrolysis of FA-Phe²-Gly-OH by DNP-CPD-Y is 10% of the value obtained with unmodified CPD-Y while for FA-Phe²-Leu-OH it was only 1% of the value obtained with CPD-Y

Figure 6. Thiolytic reaction of DNP-CPD-Y obtained after 11½ hours reaction (see Figure 5). Reaction conditions: 0.68 mg/ml CPD-Y, 0.05 M-NaH₂PO₄, 0.14 M-mercaptoethanol, pH 8.0. The spectrum between 300 and 450 nm was recorded as a function of time and in the insert $\Delta\epsilon_{341}$ is plotted versus time.

(Table II). Similarly, k_{cat}/K_m for the hydrolysis of FA-Phe²-NH₂ was approximately 50% of the value obtained with CPD-Y while for FA-Phe²-Val-NH₂ it was only 1% of the value obtained with unmodified CPD-Y (Table II). Thus, introduction of the DNP group into the active site of the enzyme affects the specificity of the enzyme with respect to the leaving group of all types of substrate such that substrates with non-bulky leaving groups relatively become better substrates of the enzyme than those with bulky leaving groups, suggesting that the DNP group prevents a favorable interaction with the latter type of substrates. These results indicate that the tyrosyl residue being modified by FDNB is located in the S_i' binding site of the enzyme.

4. DISCUSSION

Alkylation of a methionyl residue located in the S_i' binding site has previously been shown to cause drastic alterations in the leaving group specificity of the enzyme (7). In the present

paper oxidation of the same methionyl residue with H_2O_2 and modification of a tyrosyl residue similarly located in the S_1' binding site have also been found to cause alterations in the leaving group specificity and consequently, it is relevant to compare the effects of all these modifications on the enzymatic properties of CPD-Y.

CPD-Y catalyzed hydrolysis of peptide, amide and ester substrates is assumed to proceed via an acyl-enzyme intermediate in reactions where the acylation step is rate-limiting (3). In case this is generally true, k_{cat} is a measure for the rate of acylation (k_2) and K_m for K_s , the dissociation constant of the enzyme-substrate complex. Since Met-SO-CPD-Y hydrolyses ester substrates with increased k_{cat} it is suggested that the introduction of a sulfoxide group into the S_1' binding site increases the rate of acylation of ester substrates. p-NPAB-CPD-Y similarly hydrolyses ester substrates with increased k_{cat} while IAA-CPD-Y, PAB-CPD-Y, m-NPAB-CPD-Y and DNP-CPD-Y all hydrolyse ester substrates with decreased k_{cat} . The present results and those previously published (7) do not allow the causes of these effects to be established but it is probable that the introduction of the sulfoxide group changes the polarity of a portion of the S_1' binding site and a similar effect may be attributed to the p-nitrophenacylsulfonium group containing the polar nitro group.

All the alkylated derivatives of CPD-Y, i.e. IAA-CPD-Y, PAB-CPD-Y, p-NPAB-CPD-Y and m-NPAB-CPD-Y hydrolyse peptides with k_{cat} values that are only 0.5-4% of the values obtained with unmodified CPD-Y. This drastic reduction is probably not due to the loss of the methionyl residue since Met-SO-CPD-Y with the same methionyl residue oxidized hydrolyses peptides with much higher k_{cat} values, i.e. 35-50% of the values obtained with CPD-Y. Instead, it appears that the low k_{cat} values of the alkylated derivatives are due to the nature of the group introduced into these enzymes. The bulkiness of this group is not likely to be the cause since IAA-CPD-Y and PAB-CPD-Y contain groups of different bulkiness and yet hydrolyse peptides with essentially the same k_{cat} values. It is conceivable that the positively charged sulfonium ion common to all these alkylated derivatives is responsible. Such a group is not

found in Met-SO-CPD-Y and consequently, this enzyme hydrolyses peptides with much higher k_{cat} values. It appears that introduction of the DNP group in the S_1' binding site reduces k_{cat} for peptide hydrolysis somewhat more than observed for Met-SO-CPD-Y but not nearly as much as observed for the alkylated derivatives.

In general, all the described modifications increase K_m for both ester and peptide hydrolysis indicating that these groups have an adverse effect on the binding of such substrates. However, two exceptions should be mentioned: DNP-CPD-Y and PAB-CPD-Y both hydrolyse FA-Phe-OMe with decreased K_m values suggesting that the introduction of these two groups into the enzyme has a beneficial effect on the binding of this substrate. This suggests that in these two cases the combination of a substrate with a non-bulky leaving group, characterized by a relatively high K_m , and a bulky hydrophobic group occupying a position in the S_1' binding site, mimics a substrate with a bulky leaving group which is characterized by a low K_m .

Due to the drastically altered leaving group specificity some of the alkylated derivatives catalyzes deamidation reactions in much higher yields than unmodified CPD-Y (7) and these enzymes presumably will have applications in enzymatic peptide synthesis. The alkylated CPD-Y derivatives are examples of how a proteolytic enzyme can be adapted to specific purposes by protein engineering and it is probable that other modified enzymes, exopeptidases as well as endopeptidases, similarly with advantage could be applied in many reactions in place of existing unmodified enzymes. Conceivably such derivatives could be obtained not only by chemical modifications but also by site directed mutagenesis. DNP-CPD-Y and Met-SO-CPD-Y described in the present paper also exhibits an altered leaving group specificity but the alterations are not sufficient for these enzymes to be of use in deamidation reactions. However, an altered leaving group specificity implicates an altered specificity towards nucleophiles in aminolysis reactions, and DNP-CPD-Y and Met-SO-CPD-Y might conceivably have applications in this type of reactions.

Of the five methionyl residues in CPD-Y one appears to be freely accessible since it easily

reacts with H_2O_2 while a second is only partially accessible since it reacts rather slowly with H_2O_2 . The remaining three methionyl residues are probably buried in the interior of the molecule since they do not react at all. The stability of the enzyme is not affected when the accessible methionyl residue is modified, but when the partially exposed methionyl residue is modified the stability is drastically reduced. This is remarkable since only a non-bulky oxygen atom is introduced on its side-chain, and it suggests that the protein molecule is densely packed around this second methionyl residue.

Chymotrypsin (11, 12, 13, 16) and subtilisin (20) have each been shown to contain a methionyl residue which is easily oxidized by H_2O_2 . Both are located in the active site but not in the S_1' binding site. Chymotrypsin in addition contains a methionyl residue which reacts with H_2O_2 in 7 M-urea. However, the influence of this modification on the stability of the enzyme has not been studied such that the effects of a buried Met-SO group on the stability of the enzyme cannot be evaluated in this case.

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