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USE OF CIRCULAR DICHROISM TO STUDY THE INTERACTIONS OF CARBOXYPEPTIDASE A (ANSON) AND $A_{\alpha+\beta}$ WITH SUBSTRATES AND INHIBITORS

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SUMMARY

1. The phenylalanyl circular dichroism (CD) bands of peptides were used to assay peptidase activity of carboxypeptidase A (Anson) and $A_{\alpha+\beta}$ (70% A_{α}^{Leu} and 30% A_{β}^{Val}). Gly–Gly–L-Phe and Gly-L-Phe have a sharp, negative CD band at 267 nm, whereas L-phenylalanine (the optically active product) has positive CD. Thus the hydrolysis of these substrates may be measured from the CD change at 267 nm (change in $\Delta\varepsilon_M$ of about 0.04 $M^{-1} \cdot cm^{-1}$).

2. The addition of β-phenylpropionate to either carboxypeptidase $A_{\alpha+\beta}$ or A (Anson) makes the CD more positive in the region from 270 to 285 nm. Apparently this alteration results from the tyrosyl CD bands of carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1). Evidence is presented that this change arises primarily from the interaction of β-phenylpropionate with Tyr 198 (binding constant, 0.5 mM).

3. Gly–L-Phe does not produce any major alteration of the tyrosyl CD bands of carboxypeptidase $A_{\alpha+\beta}$ or A (Anson). Apparently the movement of Tyr 248 into the active site does not cause any readily measurable CD alteration, even when extensive signal averaging is carried out to achieve low noise records (peak-to-peak noise less than $5 \cdot 10^{-6}$ ΔA).

4. The binding of Gly-L-Phe, Gly-D-Phe, L-phenylalanine, or D-phenylalanine shifts the wavelength positions of the tryptophanyl CD fine structure observed in carboxypeptidase A (Anson) cooled to $-196°$. This effect may result from binding outside the active site pocket, possibly in the groove near Arg 71.

INTRODUCTION

Circular dichroism (CD) seems to be an excellent method for detecting certain

Abbreviations: CD, circular dichroism; $\Delta\varepsilon_M$, molar extinction coefficient for left circularly polarized light minus that for right circularly polarized light; ΔA, absorbance for left circularly polarized light minus that for right circularly polarized light; carboxypeptidase $A_{\alpha+\beta}$, preparation containing 70% carboxypeptidase A_{α}^{Leu} and 30% A_{β}^{Val}.

interactions between biological molecules. Recently the interactions between several enzymes and their substrates or inhibitors have been found to cause readily measurable changes in the near ultraviolet CD bands[1-5]. Since the CD spectra of macromolecules are determined by their conformations, CD may permit identifying conformation changes induced in an enzyme by substrate binding, such as would be expected from the induced fit hypothesis of enzymic catalysis[6]. Relating CD spectra to changes in protein conformation is difficult, however, because one must distinguish among four possible explanations for the altered CD: (a) the protein conformation may be altered when the substrate is bound; (b) the substrate conformation may be different in the bound state; (c) local interactions at the binding site may induce new CD bands or alter existing CD bands in either the protein or the substrate even though no conformation change occurs; and (d) long range interactions between chromophores of the protein and those of the substrate (dipole–dipole coupling)[7] may alter the CD intensities of both the substrate and the protein even in the absence of conformation changes.

It seems to us that several steps can be taken to minimize the difficulties of distinguishing among the four interpretations. First, select enzymes whose structures are known in the crystalline state. This facilitates differentiating between local and long range interactions. Secondly choose enzymes having substrates whose CD bands do not overlap the relevant bands of the enzyme. Thirdly use the tyrosyl and tryptophanyl side chain CD bands (275–300 nm) to detect changes in the enzyme, because these bands are relatively easy to identify and can be studied at high resolution by cooling to −196° (refs. 8–10). To determine the usefulness of these procedures, we have studied the CD spectra of carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1), an enzyme known to undergo a local conformation change upon substrate binding. X-ray diffraction studies of crystalline carboxypeptidase A_α have revealed that the hydroxyl group of Tyr 248 moves 12 Å upon binding of Gly-L-Tyr[11,12]. This conformation change may alter the CD spectrum of carboxypeptidase A in the region where the tyrosyl bands occur.

The present article describes the near ultraviolet CD spectra of carboxypeptidase A (Anson) and $A_{\alpha+\beta}$ after adding various substrates and inhibitors. Special attention is given to the CD fine structure of carboxypeptidase A in the region where the tyrosyl and tryptophanyl side chains have their bands (see preceding article)[13]. These measurements are made at 24, −40, and −196°. The following substrates and inhibitors are used in these studies: Gly–Gly–L-Phe, Gly–L-Phe, L-phenylalanine, Gly–L-Val, Gly–L-Leu, Gly–D-Phe, D-phenylalanine, Gly–Gly–L-Ala, L-Phe–Gly–Gly, and β-phenylpropionate. The absorption bands of these compounds do not overlap the tyrosyl and tryptophanyl bands of the enzyme in the region 275 to 300 nm. For some substrates, the extent of hydrolysis can be determined from alterations in their phenylalanyl CD bands, which occur between 250 and 270 nm[14].

MATERIALS AND METHODS

The procedures and enzymes used in this study are described in the preceding article[13]. Carboxypeptidase $A_{\alpha+\beta}$ was kindly provided by Prof. Philip Pétra and Prof. Hans Neurath. Gly–Gly–L-Phe, Gly–L-Phe, Gly–L-Val, Gly–L-Leu, Gly–Gly–L-Ala, Gly–D-Phe, L-Phe–Gly–Gly, D-phenylalanine, Gly–Gly and *N*-benzoylglycine

were obtained from Cyclo Chemical Corp., Los Angeles. L-Phenylalanine and glycine were from Sigma Chemical Corp., St. Louis, Mo. β-Phenylpropionic acid (hydrocinnamic acid) was obtained from Eastman Organic Chemicals, Rochester, N.Y. *N*-benzoyl-Gly–L-Phe was from Mann Research Laboratories, New York.

Unless otherwise indicated, the experimental uncertainty in the CD measurements is determined by the peak-to-peak noise, which may be visualized in the flat portions of each trace and which is also stated in the legends of most figures.

RESULTS

Use of CD to measure peptidase activity of carboxypeptidase A (Anson)

Our investigation of enzymatic activity and the accompanying changes in the CD of carboxypeptidase A is confined to peptide substrates and inhibitors. Carboxypeptidase A has a high affinity for peptides with an (L) aromatic residue at their C-terminus[15,16]. We chose phenylalanine to occupy this position, because its bands do not overlap the CD bands arising from the tyrosyl and tryptophanyl side chains of carboxypeptidase A. In addition, peptides containing phenylalanine have intense CD fine structure[14], which can be distinguished from the broader CD bands of carboxypeptidase A in the region from 250 to 270 nm[13]. Previous CD studies of *N*-acetyl-L-phenylalanine and L-phenylalanine[14] suggest that cleavage of a C-terminal phenylalanine residue from a peptide may cause a measurable change in the CD spectrum.

The CD spectrum of Gly-Gly-L-Phe is compared with that of its hydrolysis products (L-phenylalanine plus Gly-Gly) in Fig. 1. This tripeptide, whose CD spectrum is similar to that of *N*-acetyl-L-phenylalanine[14], has strong negative CD bands at 267 and 260.5 nm and positive bands at 257 and 251 nm. In contrast, the CD spectrum of L-phenylalanine plus Gly-Gly has only positive CD fine structure (265*,

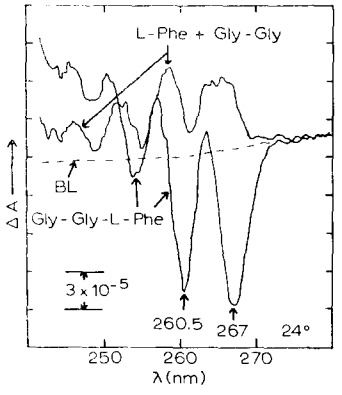

Fig. 1. Instrument traces of CD spectra of 4 mM Gly-Gly-L-Phe and of 4 mM L-phenylalanine (L-Phe) + 4 mM Gly-Gly dissolved in aqueous 0.4 M NaCl + 25 mM tris-HCl (pH 7.5) at 24°. BL designates the solvent base line. 1.0-cm path, 1.5-nm spectral half intensity band width, 0.6-sec time constant. Each trace is the average of 20 scans. Peak-to-peak noise varies from $5 \cdot 10^{-6}$ Δ*A* at 270 nm to $9 \cdot 10^{-6}$ Δ*A* at 245 nm.

* The CD spectra of Fig. 1 were recorded using the same spectral half band width as was used to record carboxypeptidase spectra; at smaller band widths, the positive CD band at 265 nm can be resolved into two separate bands[14].

258.5, and 252 nm), arising from the L-phenylalanine. The CD spectrum of the products obtained upon complete hydrolysis of Gly–Gly–L-Phe by carboxypeptidase A was identical to that shown in Fig. 1. for Gly–Gly plus L-phenylalanine. The difference in CD intensity between substrate and products is greatest at 267 nm; at room temperature $\Delta\varepsilon_M$ changes from -0.033 $M^{-1}\cdot cm^{-1}$ to $+0.011$ $M^{-1}\cdot cm^{-1}$ (spectral half band width of 1.5 nm). Thus the CD intensity at 267 nm can be used to assay peptidase activity of carboxypeptidase A.

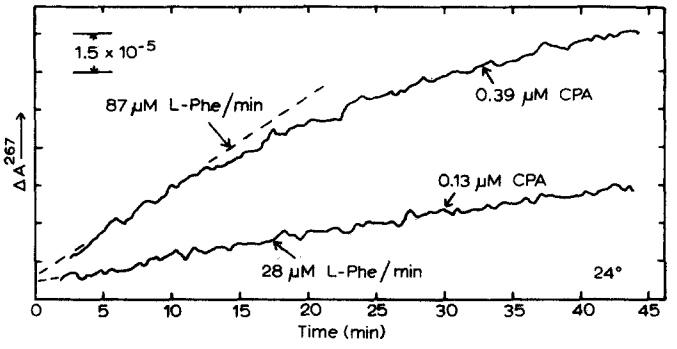

Fig. 2. Instrument traces showing CD assay of the hydrolysis of 4.0 mM Gly–Gly–L-Phe catalyzed by (CPA) carboxypeptidase A (Anson) dissolved in aqueous 25 mM Tris–HCl + 0.4 M NaCl (pH 7.5). The initial rate (— — —) was 3 times larger at 0.39 μM carboxypeptidase A (top trace) than at 0.13 μM carboxypeptidase A (bottom trace). Change in ΔA was recorded at 267 nm using a 10-sec time constant, 1-cm path length, and a 2.0-nm spectral half intensity band width. In the absence of carboxypeptidase A no change occurred in ΔA.

Fig. 2 illustrates the application of CD to measure peptidase activity, using two concentrations of carboxypeptidase A (Anson). The enzyme concentration was kept below 0.4 μM (1-cm path) so that it did not contribute significantly to the CD. At 24° the turnover number was 220 min^{-1} at 4 mM Gly–Gly–L-Phe and 320 min^{-1} at 7.8 mM Gly–Gly–L-Phe.

The hydrolysis of Gly–L-Phe by carboxypeptidase A (Anson) was also measured under the same conditions as described for Gly–Gly–L-Phe. Gly–L-Phe ($\Delta\varepsilon_M$, -0.033 $M^{-1}\cdot cm^{-1}$ at 267 nm) has the same near ultraviolet CD spectrum as does Gly–Gly–L-Phe, but is hydrolyzed much more slowly. The turnover number at 24° using Gly–L-Phe (4 mM) as substrate was only about 1 min^{-1}, in agreement with the value reported by COLEMAN AND VALLEE[17].

The reliability of the CD assay procedure was verified by comparing the rate of hydrolysis of *N*-benzoyl-Gly–L-Phe measured using both CD and the standard absorption assay[13,18]. The CD spectrum of *N*-benzoyl-Gly–L-Phe (*N*-hippuryl–L-Phe) arises from the hippuryl and the phenylalanyl chromophores. The hippuryl moiety of *N*-benzoyl-Gly–L-Phe has a broad negative CD band starting at 290 nm and dropping sharply at wavelengths below 260 nm. At 245 nm, $\Delta\varepsilon_M$ is -1.1 $M^{-1}\cdot cm^{-1}$. The phenylalanyl moiety of *N*-benzoyl-Gly–L-Phe contributes only weak fine structure between 270 and 255 nm. The products of hydrolysis (hippuric acid and L-phenylalanine) have the CD spectrum of L-phenylalanine ($\Delta\varepsilon_M$, $+0.006$ $M^{-1}\cdot cm^{-1}$ at 245 nm). The CD assay was made at 245 nm in a 2-mm path length cuvette containing the same concentrations of carboxypeptidase A (Anson) and *N*-benzoyl-

Gly–L-Phe as used in the standard absorption assay. The peptidase activity measured using CD gave values similar to those obtained from the absorption assay[13].

Effect of β-phenylpropionate upon the CD spectra of carboxypeptidase A

β-Phenylpropionate binds to carboxypeptidase A at several tyrosine sites (19–31), including the one which causes the large movement of Tyr 248 (ref. 12). Thus β-phenylpropionate seems well suited to test whether CD can be used to detect interactions involving the tyrosyl side chains of carboxypeptidase A. The inhibitor itself is not optically active, but may acquire CD bands if bound asymmetrically to carboxypeptidase A[32,33]. If this were to happen, then the near ultraviolet CD bands of β-phenylpropionate would arise from its phenyl chromophore and should resemble the bands observed in phenylalanine derivatives[14]. Bound β-phenylpropionate cannot have any CD bands at wavelengths greater than 270 nm since it does not absorb in this region.

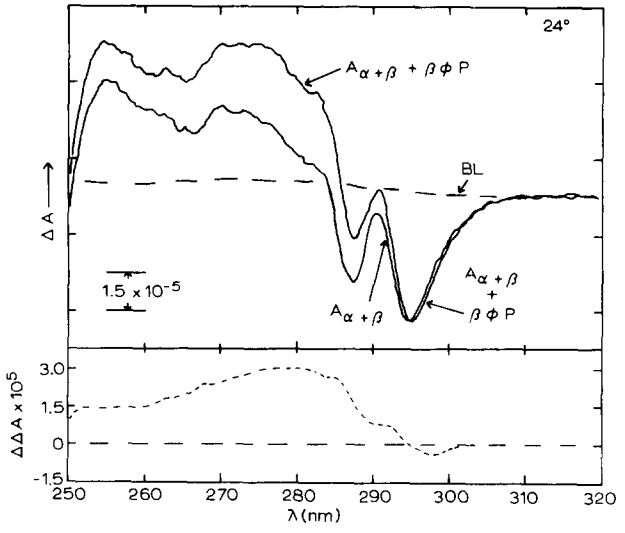

Fig. 3. Top: CD instrument trace of 12 μM carboxypeptidase $A_{\alpha+\beta}$ in the presence and absence of 1.9 mM β-phenylpropionate (βΦP). Bottom: the difference spectrum obtained by subtracting the CD spectrum of carboxypeptidase $A_{\alpha+\beta}$ from the CD spectrum of the enzyme treated with β-phenylpropionate. The solvent was aqueous 25 mM Tris–HCl + 0.4 M NaCl (pH 7.4). BL designates solvent base line. 1.0 cm path, 1-sec time constant. Each trace is the average of 16 scans. Peak-to-peak noise in these records varies from $1 \cdot 10^{-6}$ Δ*A* (295 nm) to $3 \cdot 10^{-6}$ Δ*A* (250 nm).

Fig. 3 (Top) shows the CD spectra of carboxypeptidase $A_{\alpha+\beta}$ in the presence and absence of 1.9 mM β-phenylpropionate. The difference between these two CD spectra can be used to identify which moieties are affected by the interaction between β-phenylpropionate and carboxypeptidase $A_{\alpha+\beta}$. This difference CD spectrum (bottom of Fig. 3) has an intense, broad band at about 277 nm ($\Delta\Delta\varepsilon_M$, 2.5 $M^{-1} \cdot cm^{-1}$) and two weak bands at 290 and 297 nm. The most intense part of this difference spectrum (285–265 nm) has the characteristics anticipated for an altered CD contribution from the tyrosyl side chains of carboxypeptidase A[9,10]. The minor CD alterations located above 290 nm probably involve a shift in the wavelength positions of tryptophanyl

CD bands (see section on Gly–Phe). Apparently the binding of β-phenylpropionate does not induce any readily measurable CD in its phenyl chromophore, since no major bands are observed in the difference spectrum between 270 and 250 nm.

The effect of β-phenylpropionate concentration upon CD intensification was examined using carboxypeptidase A (Anson). Fig. 4 shows the family of difference CD spectra produced by various concentrations of β-phenylpropionate. The shapes of these curves are similar for all concentrations of β-phenylpropionate, but the CD intensity at 277 nm increases as the concentration is raised from 0.06 to 5.6 mM. The difference CD spectrum for 2.2 mM β-phenylpropionate added to carboxypeptidase A (Anson) (Fig. 4) is essentially identical to that obtained for 1.9 mM β-phenylpropionate added to carboxypeptidase $A_{\alpha+\beta}$ (Fig. 3). In all these difference spectra, the major CD intensification (285–265 nm) appears to result from the tyrosyl side chains of carboxypeptidase A. The concentration dependence of the interaction(s) with a tyrosyl side chain(s) is presented in Fig. 5, using the change in $\Delta\varepsilon_M$ at 277 nm to measure alteration of the tyrosyl CD intensity of carboxypeptidase A. The curve is approximately hyperbolic with half maximal effect at about 0.5 mM β-phenylpropionate.

Effect of Gly–Phe on the CD spectra of carboxypeptidase A

Gly–L-Phe was used as a substrate because its hydrolysis is slow enough to permit accurate CD measurements. In addition, Gly–L-Phe should bind in the same way as Gly–L-Tyr and cause movement of Tyr 248 (ref. 12). Fig. 6 shows the CD spectra of carboxypeptidase $A_{\alpha+\beta}$ at different times after adding Gly–L-Phe. The

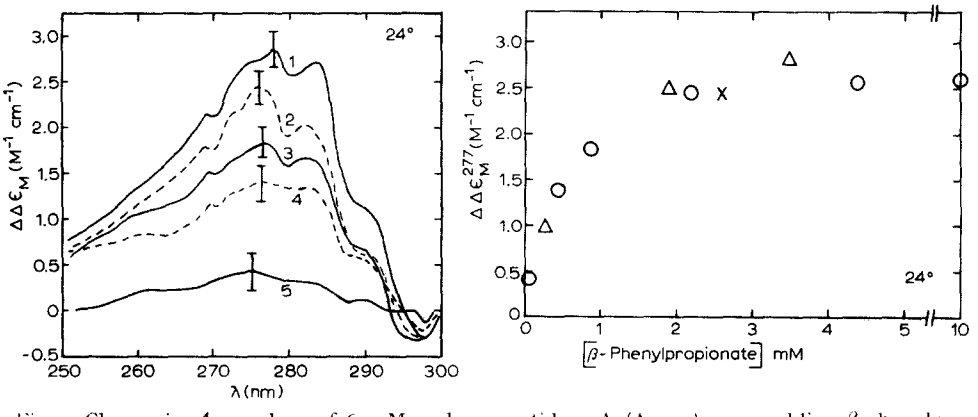

Fig. 4. Change in $\Delta\varepsilon_M$ values of 60 μM carboxypeptidase A (Anson) upon adding β-phenylpropionate (1, 5.6 mM; 2, 2.2 mM; 3, 0.86 mM; 4, 0.42 mM; 5, 0.06 mM final concentration). Solvent contained 25 mM Tris-HCl (pH 7.7) + 0.4 M NaCl dissolved in water-glycerol (1:1, by vol.). The water–glycerol solvent permitted using a 2-mm path so that the CD changes between 250 and 270 nm could be measured even at high concentrations of β-phenylpropionate. Vertical bars indicate approximate uncertainty in the $\Delta\Delta\varepsilon_M$ values. $\Delta\varepsilon_M$ is based on the molar concentration of carboxypeptidase A .

Fig. 5. Change in $\Delta\varepsilon_M$ at 277 nm as a function of β-phenylpropionate concentration. ○, 60 μM carboxypeptidase A (Anson) dissolved in water-glycerol containing 25 mM Tris–HCl (pH 7.7) + 0.4 M NaCl; ×, 15 μM carboxypeptidase A (Anson) dissolved in aqueous 25 mM Tris–HCl (pH 7.5) + 1.0 M NaCl; △, 12 μM carboxypeptidase $A_{\alpha+\beta}$ in aqueous 25 mM Tris–HCl (pH 7.4) + 0.4 M NaCl.

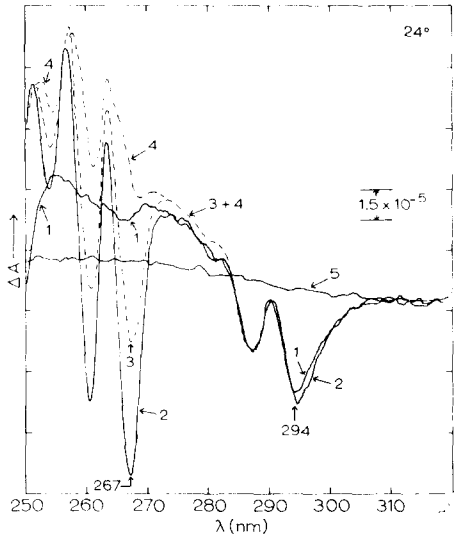

Fig. 6. CD instrument traces of 12 μM carboxypeptidase $A_{\alpha+\beta}$ before and during the hydrolysis of 4.8 mM Gly L-Phe. (1) before; (2) 0 to 1.5 h after adding Gly-L-Phe; (3) about 10 h (at 5°) afterward; (4) about 24 h (at 5°) afterward; (5) solvent base line. Curve 4 represents complete hydrolysis of the Gly-L-Phe. The solvent was aqueous 25 mM Tris-HCl (pH 7.4) + 0.4 M NaCl. 1.0-cm path, 8 scans, 1-sec time constant. Peak-to-peak noise in these records varies from $2 \cdot 10^{-6}$ ΔA (295 nm) to $4 \cdot 10^{-6}$ ΔA (250 nm).

extent of hydrolysis of the substrate was followed from the altered intensity of its CD fine structure. Gly-L-Phe has negative CD bands at 267 and 260.5 nm, which become more positive as the products (L-phenylalanine and glycine) build up, *e.g.* see Fig. 1.

Since neither Gly-L-Phe nor L-phenylalanine has any CD above 273 nm, the long wavelength region was used to search for possible changes in the conformation of carboxypeptidase $A_{\alpha+\beta}$. Addition of 4.8 mM Gly-L-Phe to carboxypeptidase $A_{\alpha+\beta}$ caused a minor alteration in the tryptophanyl CD band[13] at 294 nm (Fig. 6). As the hydrolysis of Gly L-Phe proceeded, the CD became slightly more positive in the region from 273 to 284 nm ($\Delta A \varepsilon_M$, 0.6 $M^{-1} \cdot cm^{-1}$ at 281 nm).

More extensive experiments were carried out using carboxypeptidase A (Anson). Addition of 4 mM Gly-L-Phe to carboxypeptidase A (Anson) at 24° altered the CD spectrum in the same way as described above for carboxypeptidase $A_{\alpha+\beta}$, except that the positive intensification extended from 273 to 290 nm ($\Delta A \varepsilon_M$, 0.9 $M^{-1} \cdot cm^{-1}$ at 281 nm). A 5-fold higher concentration of Gly-L-Phe (20 mM) at 5° did not further enhance the CD changes observed for carboxypeptidase A (Anson) from 280 to 300 nm. Apparently the lack of a major CD alteration is not due to poor binding of Gly-L-Phe by carboxypeptidase A. This conclusion is supported by the findings of COLEMAN AND VALLEE[17] that Gly-L-Phe binds readily even though the hydrolysis rate is slow.

To obtain better resolution of the tyrosyl and tryptophanyl CD bands of carboxypeptidase A, further studies using Gly L-Phe were carried out at lower temperatures. At −40° the effects of Gly-L-Phe upon carboxypeptidase A (Anson)

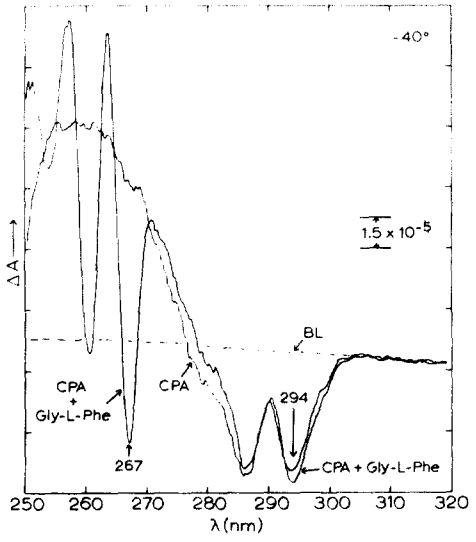

Fig. 7. CD instrument traces of 12.6 μM carboxypeptidase A (Anson) (CPA) in the presence and absence of 4 mM Gly–L-Phe at −40°. The water–glycerol solvent contained 25 mM Tris–HCl (pH 7.5) + 0.4 M NaCl. BL designates the solvent base line. 1.0-cm path, 20 scans. Peak-to-peak noise in these records varies from $2 \cdot 10^{-6}$ ΔA (295 nm) to $4 \cdot 10^{-6}$ ΔA (250 nm).

were more pronounced than those observed at 24° (Fig. 7): the 294-nm band was slightly intensified and red-shifted by 0.3 nm, and the CD became more positive between 270 and 287 nm ($\Delta\Delta\varepsilon_M$, 1.5 $M^{-1} \cdot cm^{-1}$ at 277 nm). Cooling to −196° permitted a more detailed examination of the effects of Gly–L-Phe upon the trypto-

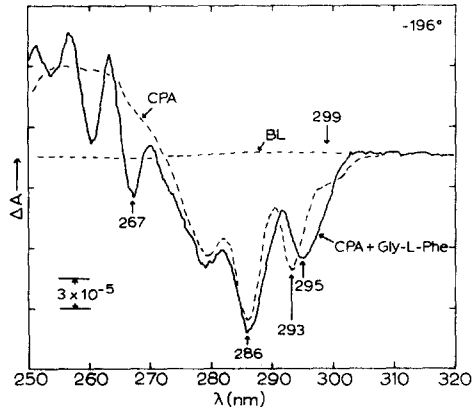

Fig. 8. ———, CD instrument trace of 80 mM Gly–L-Phe + 1.0 mM carboxypeptidase A (Anson) at −196°. — — —, CD spectrum of 1.0 mM carboxypeptidase A (Anson) (CPA) at −196°. The water–glycerol solvent contained 25 mM tris-HCl (pH 7.4) + 0.4 M NaCl. BL designates solvent base line. 0.12 mm path, 20 scans. The peak-to-peak noise in these records varies from $2 \cdot 10^{-6}$ ΔA (295 nm) to $5 \cdot 10^{-6}$ ΔA (250 nm). The wavelength shifts in the bands at 293 and 299 nm greatly exceed the experimental uncertainty in peak positions for each record (about ± 0.5 nm in experiments on 18 different frozen solutions of carboxypeptidase). The intensity of the negative CD band at 267 nm indicates that less than 15% of the Gly–L-Phe was hydrolyzed prior to these measurements.

phanyl CD bands of carboxypeptidase A (Anson). Gly–L-Phe shifted one tryptophanyl CD band[13] from 293 to 295 nm and caused another tryptophanyl CD at 299 nm to disappear or shift to shorter wavelengths (Fig. 8). These alterations in the tryptophanyl CD bands are consistent with the observations at higher temperatures (Figs. 6 and 7). In the 270 to 290 nm region, however, the effect of Gly–L-Phe upon carboxypeptidase A (Anson) at −196° differed from that described above. Addition of Gly–L-Phe to carboxypeptidase A (Anson) at −196° made the CD slightly more negative between 270 and 290 nm (Fig. 8). This difference in results between 24° and −196° may be due to the great intensification of the tryptophanyl CD bands[13] at −196°, which may mask the CD alterations involving other protein moieties.

The absorption spectra of carboxypeptidase A (Anson) at −196° was also altered by adding Gly–L-Phe. The tryptophanyl absorption band at 302 nm was slightly better resolved and the tryptophanyl band at 290.5 nm was less well-resolved for the Gly–L-Phe–carboxypeptidase A complex than for the free enzyme**.

The effects of the enantiomorphic compound Gly–D-Phe were also examined. At 24° Gly–D-Phe (16 mM) caused the same alterations in the CD spectrum of carboxypeptidase A (Anson) as did Gly–L-Phe in the region above 273 nm. At −196° the addition of 50 mM Gly–D-Phe to 1.4 mM carboxypeptidase A (Anson) gave the same changes in the tryptophanyl CD bands (290 to 300 nm) as shown for Gly–L-Phe in Fig. 8.

Effects of phenylalanine on the CD spectra of carboxypeptidase A (Anson)

Fig. 9 shows the CD spectra of carboxypeptidase A (Anson) at 24° after adding

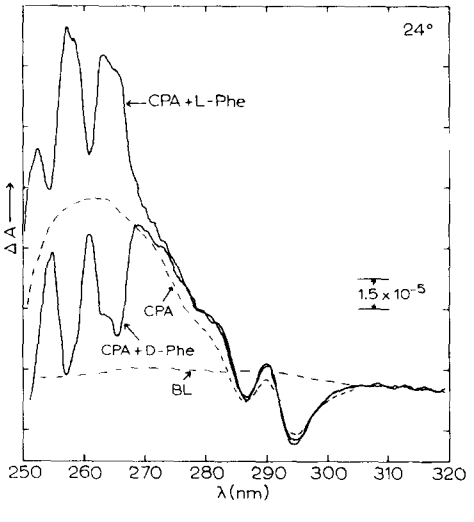

Fig. 9. Effect of 4 mM L- or D-phenylalanine (— ——, Phe) upon the CD spectrum of 9.2 μM carboxypeptidase A (Anson) (- · — —, CPA). Solid lines are actual instrument traces. The aqueous solvent contained 0.4 M NaCl + 25 mM Tris–HCl (pH 7.5). BL designates the solvent base line. 1.0-cm path, 20 scans. The peak-to-peak noise in the records varies from $1 \cdot 10^{-6}$ ΔA (295 nm) to $3 \cdot 10^{-6}$ ΔA (250 nm).

** The absorption spectrum of carboxypeptidase A (Anson) at −196° is shown in Fig. 4 of the preceding article[13].

either L- or D-phenylalanine. The phenylalanine CD bands have mirror image symmetry about the spectrum of carboxypeptidase A between 250 and 270 nm. Either of these compounds causes the same alterations in the CD spectrum of carboxypeptidase in the region above 275 nm. The tryptophanyl CD band at 294 nm is slightly intensified, and the CD between 270 and 290 nm is more positive ($\Delta M\varepsilon_M$, 1.3 $M^{-1} \cdot cm^{-1}$ at 282 and 277 nm). At −196° the CD spectrum of carboxypeptidase A (Anson) is altered by either D- or L-phenylalanine in the same manner as described above for Gly–L-Phe, *i.e.*, a 2-nm red shift of the peak to 295 nm and loss of the 299-nm CD band of the tryptophanyl side chains.

Effects of other compounds on the CD spectra of carboxypeptidase A (Anson)

Owing to the rapid hydrolysis of Gly–Gly–L-Phe, it was not possible to obtain a low noise CD spectrum of the Gly–Gly–L-Phe–carboxypeptidase A complex throughout the entire near ultraviolet region. However, by examining only a 30-nm wavelength interval (310–280 nm) the Gly–Gly–L-Phe–carboxypeptidase A complex could be examined. The addition of 20 mM Gly–Gly–L-Phe to 12 μM carboxypeptidase A (Anson) at 5° caused some intensification along the red edge of the tryptophanyl CD band at 294 nm, just as found for the addition of Gly–L-Phe at 5 and 24°. Meaningful CD measurements were not possible at −196° using Gly–Gly–L-Phe with carboxypeptidase A. Owing to the low solubility of Gly–Gly–L-Phe, the extent of its hydrolysis prior to freezing was indeterminable.

CD spectra were recorded at −196° using L-Phe–Gly–Gly. This peptide altered the tryptophanyl CD bands of carboxypeptidase A (Anson) in different ways than did the compounds having a C-terminal phenylalanine. L-Phe–Gly–Gly (80 mM) caused the 299-nm CD band of carboxypeptidase A (Anson) to intensify slightly instead of disappear. In addition, the red shift of the 293-nm CD band was only half that observed with Gly–L-Phe; and its intensity decreased by 30%.

The effects of compounds lacking an aromatic group were also examined. Gly–L-Leu is another substrate whose hydrolysis rate is slow even though it binds readily to carboxypeptidase A[17]. At 24° the addition of 24 mM Gly–L-Leu did not alter the CD spectrum of carboxypeptidase A (Anson) above 270 nm. Likewise, at −196° adding Gly–L-Leu (110 mM) did not affect the tyrosyl and tryptophanyl CD bands of carboxypeptidase A (Anson). Most of the substrate should have remained unhydrolyzed in these experiments, because the turnover number for Gly–L-Leu (0.7 min^{-1}) is less than that for Gly–L-Phe[17].

In addition, the CD spectrum of carboxypeptidase A (Anson) was recorded at −196° after adding glycine (60 mM), Gly–L-Val (140 mM) or Gly–Gly–L-Ala (60 mM). None of these compounds altered the CD spectrum above 270 nm.

DISCUSSION

The CD bands of substrates may be used to measure the peptidase activity of carboxypeptidase A. A peptide containing a C-terminal phenylalanine residue has a much different CD spectrum from that of L-phenylalanine, which is liberated during hydrolysis (Fig. 1). Thus the sharp phenylalanyl CD band at 267 nm permits assaying the rate of hydrolysis of substrates, such as Gly–L-Phe and Gly–Gly–L-Phe, whose absorption of unpolarized light is not much affected by hydrolysis. Changes in the

near ultraviolet CD bands of peptides having tyrosine or tryptophan as the C-terminal residue may permit measuring their hydrolysis also. For other peptides their CD bands at shorter wavelengths may be altered during hydrolysis.

CD also permits studying the interactions between carboxypeptidase A and various substrates and inhibitors. The substrates and inhibitors used in this study do not have absorption or CD bands which overlap the tyrosyl and tryptophanyl CD bands (275–300 nm) of carboxypeptidase A[13]. Therefore, any alterations of CD intensity between 275 and 300 nm must arise from the protein CD bands being affected by the interaction.

Adding β-phenylpropionate to carboxypeptidase A produces a major CD intensification centered about 277 nm (Figs. 3 and 4), just as would be expected from an altered tyrosyl CD band[9,10]. This observation can be related to specific tyrosine residues of carboxypeptidase A. Much chemical evidence indicates that the binding by carboxypeptidase A of the competitive inhibitor β-phenylpropionate protects two functional tyrosine residues[12,19–31]. On the basis of their X-ray data, LIPSCOMB *et al.*[12] proposed that two β-phenylpropionate binding sites are located at the active site. At one site, binding induces a large movement of the side chain of Tyr 248; the second site is near Tyr 198 (ref. 12). In crystalline carboxypeptidase A either of these sites may be occupied when the surrounding medium contains 10 mM β-phenylpropionate[29].

This concentration of β-phenylpropionate is not necessarily required for binding to carboxypeptidase A in solution. A comparison of the inhibition of peptidase activity in crystals and solutions indicates that β-phenylpropionate binds much more effectively to carboxypeptidase A in solution than in the crystal. The inhibition constant for β-phenylpropionate decreases from 10 mM for crystals to 0.1 mM for dissolved carboxypeptidase A[34]. Apparently the inhibition of peptidase activity represents the interaction of β-phenylpropionate with the site involving Tyr 248, because only this tyrosine residue is essential for peptidase activity[11,12]. When carboxypeptidase A is dissolved in a solution containing 1–2 mM β-phenylpropionate, probably both the site involving Tyr 248 and the site near Tyr 198 may be occupied. Evidently the β-phenylpropionate binding previously measured for carboxypeptidase A solutions[26] may involve both these sites, since no distinction between them was made until more recently[29]. Additional β-phenylpropionate binding sites have been observed[29], having binding constants of about 10 mM for the dissolved enzyme[30]. These weaker binding sites also involve tyrosine residues[30].

In our CD experiments with both carboxypeptidase $A_{\alpha+\beta}$ and A (Anson), β-phenylpropionate produced CD alterations having the wavelength position (277 nm) and shape expected for an interaction with a tyrosyl side chain (Figs. 3 and 4). This enhanced CD was readily observed only at inhibitor concentrations above 0.2 mM and was half maximal at 0.5 mM (Fig. 5). These concentrations are appreciably above those required to inhibit peptidase activity in solution[26,34]. Apparently the CD intensification caused by β-phenylpropionate at 277 nm does not result primarily from the rearrangement of Tyr 248. Instead, the CD enhancement seems to arise from the binding of β-phenylpropionate near Tyr 198. Thus the binding constant for this site may be estimated to be about 0.5 mM (Fig. 5).

Next we shall examine the binding of phenylalanine, Gly-Phe, and Gly-L-Leu. Gly-L-Phe is an analog of Gly-L-Tyr, which was used as a substrate for the X-ray

studies[11,12]. Gly–L-Phe is expected to bind with its phenyl ring in the active site pocket, causing Tyr 248 to swing into its functional position[12]. Apparently Gly–L-Leu should bind in the same way[17]. Although (LL) dipeptides have only this single binding site in crystalline carboxypeptidase A[12], no experimental evidence precludes additional binding sites outside the pocket when carboxypeptidase A is dissolved in solution[26,35]. Gly–D-Phe probably does not bind within the active site pocket***, since a similar compound (*N*-acetyl-D-phenylalanine) is not bound there[35]. Both D- and L-phenylalanine, however, do bind at the pocket, although only the L-isomer causes the rearrangement of Tyr 248 (refs. 12, 29).

Apparently the movement of Tyr 248 into the active site does not in itself cause any measurable alteration in the CD spectrum of carboxypeptidase A. For example, the addition of Gly–L-Leu did not affect the CD bands of carboxypeptidase A in the region of the tyrosyl CD bands (270–290 nm). The binding of Gly–L-Phe produced only a minor CD intensification between 270 and 290 nm (Figs. 6 and 7). The latter alteration seems more related to the binding of a phenyl ring than to the movement of Tyr 248. Both Gly–D-Phe and D-phenylalanine (Fig. 9) also produced the CD intensification (270–290 nm) even though their binding should not reorient Tyr 248.

What is the origin of the enhanced CD (270–290 nm) caused by the compounds containing phenylalanine? With the exception of Tyr 248, there are no aromatic residues near the active site pocket in the region of (LL) dipeptide binding[12]. When Tyr 248 swings into its functional position, the center-to-center separation between this tyrosyl ring and a phenyl ring bound in the pocket decreases from 12 Å to 7 Å. This shorter separation tends to favor an interaction between the near-ultraviolet transitions of Tyr 248 and the far-ultraviolet transitions of the bound phenyl ring; *i.e.* dipole–dipole coupling may give rise to altered CD between 270 and 290 nm. This mechanism is much more effective for substrates having an aromatic ring than for peptides with aliphatic side chains[10], *e.g.* Gly–L-Leu. Although interactions at the active site may affect the CD upon binding of some phenylalanine compounds, the CD intensification (270–290 nm) caused by Gly–D-Phe and D-phenylalanine may involve a different binding site (see below).

The question arises why the movement of Tyr 248 *per se* does not cause a detectable change in the 270 to 290 nm region of the CD spectrum of carboxypeptidase A. One difficulty is that the tryptophanyl bands dominate the CD spectrum of carboxypeptidase A above 275 nm[13]. In addition, there are 18 other tyrosyl side chains[12], which may also have CD bands. Thus any CD change due to Tyr 248 must be measured against a much larger background CD from many other aromatic residues. The experimental uncertainties ($|\Delta\Delta\varepsilon_M|$, about 0.3 $M^{-1}\cdot cm^{-1}$) are comparable to the $\Delta\varepsilon_M$ values of many tyrosyl model compounds[9,10]. Apparently the interaction of β-phenylpropionate at Tyr 198 is readily observed only because the change is especially large ($\Delta\Delta\varepsilon_M$ about 2.5 $M^{-1}\cdot cm^{-1}$).

Other techniques using modified carboxypeptidases having visible CD bands may offer a means to detect the movement of Tyr 248. Both cobalt carboxypeptidase[36] and arsanilazocarboxypeptidase[37] have visible CD bands that are altered by binding of Gly–L-Tyr.

*** Lipscomb has found from model building that Gly–D-Phe does not bind in the active site pocket of crystalline carboxypeptidase A (personal communication).

As our final point, we consider the pronounced wavelength shifts of the tryptophanyl CD bands observed at −196° after the binding to carboxypeptidase of Gly-L-Phe and other compounds containing a phenyl ring (Fig. 8). Interestingly the alterations of the tryptophanyl CD bands are accompanied by small changes in the tryptophanyl absorption, which we observed at −196° and FUJIOKA AND IMAHORI[38] found in difference spectra at 22°. These alterations do not seem directly related to the catalytic mechanism. One substrate, Gly-L-Leu, did not give the effects, whereas a non-substrate, Gly–D-Phe, did. The similarity of the alterations in tryptophanyl CD fine structure caused by both optical isomers of Gly–Phe and of phenylalanine suggests that the effects may arise from binding at the same site. Evidently an ancillary binding site may be involved, since Gly–D-Phe does not seem to bind within the active site pocket of carboxypeptidase A (see above). Furthermore, the active site pocket is some distance from the nearest tryptophan residue (10 Å from the center of a phenyl ring bound in the pocket to the center of the indolyl ring of Trp 147).

On the basis of model building, LIPSCOMB[39] has suggested that Arg 71 and Arg 127 may function as temporary binding sites for peptides passing down the groove into the active site pocket. Binding in the groove to Arg 71 may explain the mechanism by which the tryptophanyl CD bands of carboxypeptidase A are altered (Fig. 8). If the carboxyl group of phenylalanine or Gly–Phe were electrostatically bound to the guanidinium group of Arg 71, then their phenyl ring could be located near both Trp 73 and Trp 126 (6 to 7 Å between ring centers). The altered tryptophanyl CD bands may result either from a local conformational change around Trp 73 and Trp 126 or from dipole–dipole coupling[7] involving these indolyl rings and the bound compound.

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