BBA 66320

EFFECT OF TEMPERATURE UPON THE CONFORMATIONS OF CARBOXYPEPTIDASE A (ANSON), A_γ^{Leu}, A_γ^{Val}, AND $A_{\alpha,\beta}$

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(Received December 14th, 1970)

SUMMARY

1. The conformations of several bovine carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1) preparations were compared using the circular dichroism (CD) spectra from 200 to 330 nm. Carboxypeptidase A_γ^{Val}, A_γ^{Leu} and $A_{\alpha,\beta}$ (70% A_α^{Leu} and 30% A_β^{Val}) have nearly identical CD spectra. After considering the origin of the various CD bands, it is suggested that these species have similar backbone conformations and similar environments for their tryptophanyl side chains.

2. The CD spectrum of carboxypeptidase A (Anson) differs in one respect from that of the homogeneous species (A_γ^{Val} and A_γ^{Leu}). In the Anson preparation the broad, positive CD band between 255 and 270 nm is about twice as intense as that in either carboxypeptidase A_γ^{Val} or A_γ^{Leu}. The intensification of this CD band in the Anson preparation may possibly be due to an impurity. Alternatively the conformation of the carboxypeptidase A_γ molecules may be altered during their isolation from the Anson preparation.

3. The effect of temperature upon the conformations of carboxypeptidase A_γ^{Val}, A_γ^{Leu}, $A_{\alpha,\beta}$ and A (Anson) was examined by recording CD spectra between 24 and −196°. The far ultraviolet CD bands of these carboxypeptidase preparations are not altered by cooling, which suggests that the peptide backbone conformation is relatively rigid. In contrast, the CD fine structure arising from the tryptophanyl side chains is greatly intensified upon cooling the various preparations. This behavior results because the tryptophanyl side chains of carboxypeptidase A exist in multiple conformations at 24°. Cooling shifts more side chains into the conformer having the lowest energy. Apparently some side chains of carboxypeptidase A possess appreciable motility at room temperature.

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 $A\alpha^{Leu}$ and 30% A_β^{Val}.

INTRODUCTION

Circular dichroism[1-4] (CD) and nuclear magnetic resonance[5,6] spectra at varying temperatures have revealed that amino acids and dipeptides possess multiple conformers in solution. In many proteins, on the other hand, the extensive interactions between amino acid side chains apparently tend to stabilize a single conformer in the native state. X-ray studies of crystals indicate a unique conformation for each protein, although some motility may exist for certain side chains[7]. There is evidence suggesting that proteins may have greater motility in solution than in the crystalline state[7,8]. Some conformational motility is essential for the catalytic activity of carboxypeptidase A[9,10] and perhaps other enzymes[11]. Even in the absence of substrates, an enzyme may have several conformations that are readily interconverted. When motility exists, temperature variations should alter the distribution of conformers in accordance with the Boltzmann factor[12]. Such studies need to be made at low temperatures to avoid complications arising from heat denaturation of enzymes.

This article describes the conformational changes induced in bovine carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1) by cooling. The extent of conformational alteration was followed by recording CD spectra in the temperature range of 24 to −196°. The near ultraviolet CD spectra of carboxypeptidase A reveal fine structure CD bands arising from tryptophan and possibly also tyrosine residues. These bands permit measuring the effect of temperature upon the orientation of the tryptophanyl and perhaps the tyrosyl side chains in carboxypeptidase A. The far ultraviolet CD spectra are used to examine possible alterations in the peptide backbone.

An additional objective of this investigation is to compare the CD spectra of several carboxypeptidase A preparations available to us. The different procedures used to activate procarboxypeptidase A give a variety of heterogeneous carboxypeptidases[13]. Carboxypeptidase A (Anson)[14] contains mainly A_γ^{Val} (40%) and A_γ^{Leu} (30%)[15], whereas carboxypeptidase A (Cox)[16] is largely A_α^{Val} (42%) and A_α^{Leu} (34%)[17]. We have performed variable temperature CD measurements on 4 carboxypeptidase preparations: A (Anson), A_γ^{Val}, A_γ^{Leu}, and a purified fraction of A (Cox).

EXPERIMENTAL SECTION

Instrumentation

Absorption spectra and peptidase kinetics were obtained with a Cary Model 15 spectrophotometer. CD measurements were made with an extensively modified[2,18] prototype of the Beckman Far UV-CD spectrophotometer using a 75-W high pressure Xenon arc. A computer of average transients[19] stored the signal while the spectra were scanned repetitively at 0.3 nm per sec. This signal averaging permitted obtaining low-noise records having good resolution, even for the weak CD bands of carboxypeptidase A. A time constant of one second and a spectral half intensity band width of less than 1.6 nm were used in most cases. All the CD spectra at −196° were recorded using a 0.6-sec time constant. The CD intensity was calibrated with an aqueous solution of (+)-10-camphorsulfonic acid ($\Delta\varepsilon_M = 2.2\ M^{-1}\cdot cm^{-1}$ at 290 nm (see ref. 20)).

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.

Low temperature procedures

Measurements were made over the temperature range of 24 to −196°. Frozen solutions (glasses) were examined at −196° in a spectroscopic Dewar filled with liquid nitrogen, as has been described previously in more detail[1,2,18]. Carboxypeptidase A was dissolved in a water–glycerol (1:1, by vol.) solution containing 0.4 M NaCl and 25 mM Tris–HCl at pH 7.5. The cuvette (0.12 mm path) containing this solution was plunged into the spectroscopic Dewar filled with liquid nitrogen. Even this rapid freezing (5–10 sec) of the solution gave good glasses for CD measurements[2]. Tests described elsewhere[2] showed that no artifacts existed under the conditions used to record low temperature CD spectra.

Variable temperature spectra of solutions (24 to −40°) were obtained with a closed cuvette (0.1- to 10-mm path) positioned in a spectroscopic Dewar cooled by blowing cold nitrogen gas over the cuvette holder[1]. The water–glycerol buffer (0.4 M NaCl–25 mM Tris–HCl, pH 7.5) remained a single phase at temperatures down to −40°. No measurements were made below −40° because ice particles separated from the water–glycerol buffer upon standing at these lower temperatures.

The CD spectra of carboxypeptidase A in glasses at −196° gave much greater resolution than the spectra of solutions, which necessarily had to be recorded at much higher temperatures. The absolute intensities of the CD spectra of glasses, however, are somewhat less accurate than those of solutions. For CD signals as small as those of carboxypeptidase A, the exact position of the base line (frozen solvent) has some uncertainty at the short wavelength end of the CD spectrum. The long wavelength side is not uncertain because the leading edges of the base line and of the carboxypeptidase spectrum can be superimposed in regions where no CD bands exist, *i.e.* 320–330 nm. An additional disadvantage of the measurements at −196° is that short path lengths must be used to prevent CD artifacts in glasses[1,2]. Only carboxypeptidase A (Anson) was sufficiently soluble (see below) to permit optimal measurements at −196°.

Most spectra were recorded on solutions cooled in the variable temperature system. The spectral changes upon cooling were verified in duplicate experiments, and the spectral changes were reversed by rewarming solutions to room temperature. The volume contraction of the water–glycerol solutions (J. HORWITZ, personal communication), which was less than 3% at −40° and less than 10% at −196°, was neglected in calculating the concentrations of carboxypeptidase A at low temperatures.

Enzymes

Bovine pancreatic carboxypeptidase A (Anson) was purchased from Worthington Biochemical Corp. (Freehold, N.J.) as an aqueous suspension of twice-crystallized enzyme (Lot CoA 8KA). Bovine carboxypeptidase A_γ^{Val} and A_γ^{Leu} and a chromatographically purified fraction of A (Cox) were kindly provided by Prof. Philip Pétra and Prof. Hans Neurath. The latter sample (Fraction II in Fig. 2 of ref. 17), which contained 70% carboxypeptidase A_α^{Leu} and 30% carboxypeptidase A_β^{Val}, will be

designated here as $A_{\alpha+\beta}$. Aliquots drawn from these stock suspension were washed with triple-distilled water and centrifuged at 5° to sediment the crystals. For the Anson preparation this procedure was repeated 3 or 4 times for each aliquot to ensure removal of the toluene preservative. The crystals were then dissolved in the appropriate solvent (see below). These solutions were stored at 5° and were never used for longer than three days.

Three solvent systems were used for the carboxypeptidase A preparations. For studies at room temperature 25 mM Tris–HCl (pH 7.5) and either 0.4 or 1 M NaCl were dissolved in triple-distilled water; for measurements below 0° the medium contained 25 mM Tris–HCl (pH 7.5) and 0.4 M NaCl dissolved in water–glycerol (1:1, by vol.). The low temperature experiments were made using the 0.4 M NaCl to minimize the possibility of polymer formation[21].

Both carboxypeptidase A (Anson) and $A_{\alpha+\beta}$ dissolved readily (up to 12 μM) in the aqueous 0.4 M NaCl–25 mM Tris-HCl buffer. These preparations were even more soluble in water–glycerol (1:1, by vol.) containing 0.4 M NaCl–25 mM Tris (up to 2 mM for carboxypeptidase A (Anson) and up to 0.5 mM for $A_{\alpha+\beta}$). In contrast to carboxypeptidase A (Anson) and $A_{\alpha+\beta}$, carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ dissolved poorly (up to 4 μM) in 0.4 M NaCl–25 mM Tris (pH 7.5). By raising the NaCl concentration to 1 M, the solubility of carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ was increased sufficiently (7-fold) to permit CD measurements under optimal conditions.

The concentrations of all carboxypeptidase solutions were calculated from the absorbance at 278 nm (ε_M, $6.42 \cdot 10^4$ (see refs. 22 and 23)).

Assay of peptidase activity

The hydrolysis of *N*-benzoyl-glycyl-L-phenylalanine by carboxypeptidase A was determined from the change in absorbance at 254 nm, using a minor modification of the procedures described by other workers[24–26]. The assay was initiated by adding 0.02 μM carboxypeptidase A (Anson) to 1 mM substrate dissolved in one of the three solvent systems described above. *N*-Benzoyl-glycyl-L-phenylalanine has ε_M of 2600 at 254 nm; and, under the conditions used, ε_M at 254 nm increases by 360 upon complete hydrolysis. The turnover number was calculated from the initial velocities during the first 5 min of hydrolysis ($< 15\%$ of substrate consumed).

Materials

N-Benzoyl-glycyl-L-phenylalanine was obtained from Mann Research Laboratories, N.Y. Glycerol was spectroquality from Matheson, Coleman and Bell, Los Angeles. Guanidine–HCl was prepared from guanidine carbonate (Eastman) by the Kawahara–Tanford procedure[27].

RESULTS

CD spectra of carboxypeptidase A (Anson) at 24°

The various bands in the CD spectra of carboxypeptidase A give information about different aspects of conformation. The far ultraviolet CD bands at 209 and 230 nm in the native enzyme (Fig. 1) reflect primarily the conformation of the peptide backbone[28]. For example, 8 M guanidine–HCl abolishes these CD bands (Fig. 1). Thus the CD bands at 209 and 230 nm provide a measure of whether cooling

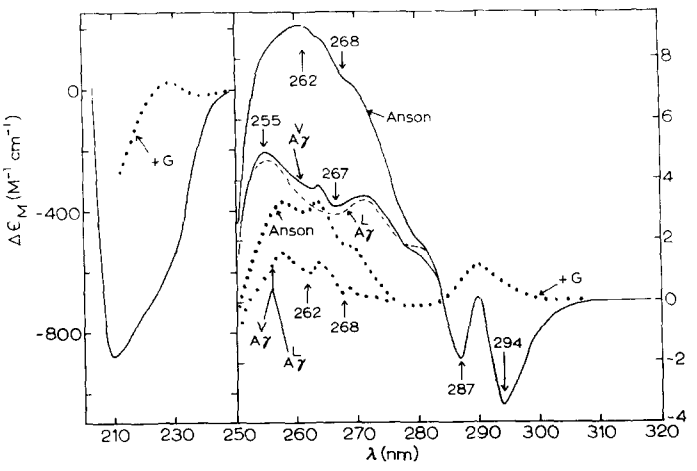

Fig. 1. Comparison of CD spectra of carboxypeptidase A (Anson), $A_\gamma{}^{Val}$, and $A_\gamma{}^{Leu}$ at 24°. $A_\gamma{}^{V}$, $A_\gamma{}^{L}$, and Anson are used to identify carboxypeptidase $A_\gamma{}^{Val}$, $A_\gamma{}^{Leu}$, and A (Anson), respectively. +G (●●●) designates enzymes dissolved in 8 M guanidine HCl (pH 6.5). —— or · · · indicates native enzymes dissolved in aqueous 25 mM Tris–HCl + 1 M NaCl (pH 7.5). The CD spectrum of the Anson material was unchanged when the NaCl concentration was decreased to 0.4 M. $\Delta\varepsilon_M$ is based on the molar concentration of carboxypeptidase A.

alters the degree of organization in the peptide backbone of carboxypeptidase A.

In the near ultraviolet region the native enzyme possesses a broad positive band at 262 nm and negative CD bands at 287 and 294 nm. The intensities of these near ultraviolet CD bands also depend upon a highly ordered protein conformation, since 8 M guanidine–HCl greatly diminishes the intensities of these bands (Fig. 1).

The 294- and 287-nm CD bands of native carboxypeptidase A reflect mainly the orientation of tryptophanyl side chains. The negative CD fine structure at 294 nm must arise from tryptophanyl side chains, because none of the other moieties have fine structure bands at this wavelength[2]. The negative CD fine structure at 287 nm is located in a region where either tyrosyl or tryptophanyl bands may occur. At 24°, however, tyrosyl CD bands are not as sharp as the 287-nm CD band and do not have this shape[3,4]. Apparently the 287-nm CD band also results mainly from tryptophanyl side chains.

The source of the broad positive CD band at 262 nm in the native carboxypeptidase A (Anson) is uncertain. The single disulfide bond of carboxypeptidase A[9,29,30] may be a major contributor, since cystine has a similar near ultraviolet CD band[31,32]. The total CD intensity of the 262-nm band of native carboxypeptidase A (Anson) is, however, appreciably larger than the disulfide bands identified in other proteins[3,33,34]. Some contributions from tryptophanyl and tyrosyl side chains are difficult to exclude[2,3]. The broad positive CD band centered at 262 nm cannot arise from the phenylalanyl side chains, since the phenylalanyl CD bands have different characteristics[1,35]. The red side of the longest wavelength phenylalanyl CD band (about 268 nm) always moves sharply away from the background CD due to other residues. The minor wiggle at 268 nm in the native Anson preparation (Fig. 1) may result from a very weak negative phenylalanyl CD band superimposed on the broad positive CD band. Treatment with guanidine–HCl makes the negative fine structure

at 268 nm more obvious and brings out additional negative fine structure at 262 nm, superimposed upon a diminished positive CD background (Fig. 1). Apparently the phenylalanyl CD bands of carboxypeptidase A (Anson) may be slightly enhanced after denaturation, as has been observed previously for another protein[35].

CD spectra of carboxypeptidase $A_\gamma{}^{Val}$ *and* $A_\gamma{}^{Leu}$ *at 24°*

Since carboxypeptidase A (Anson) is a heterogeneous mixture of carboxypeptidases[15], we investigated the CD spectra of the two major species in this preparation ($A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$). A comparison of the CD spectra of carboxypeptidase A (Anson), $A_\gamma{}^{Val}$, and $A_\gamma{}^{Leu}$ is shown in Fig. 1. Both carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ have similar CD spectra. All 3 preparations have identical CD bands in the far ultraviolet and above 285 nm. In the range from 250 to 280 nm, however, carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ have much weaker CD than does the Anson material. Carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ seem to have a negative CD band (268 to 260 nm) superimposed upon a larger positive band extending from 280 to 250 nm. This negative CD band has the characteristics expected for phenylalanine residues in which all 3 progressions of bands are negative and have comparable intensities[1,35]. Even after compensating for the presence of this negative CD band, the positive CD band is only about half as intense in carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ as in the Anson material.

This major difference in the CD bands of these preparations warranted further investigation, since carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$ comprise 70% of the protein isolated from the Anson material[15]. The 3 carboxypeptidase preparations were treated with 8 M guanidine–HCl to eliminate the intramolecular interactions which stabilized the original conformation. This treatment altered the shape of the negative phenylalanyl CD bands in carboxypeptidase $A_\gamma{}^{Val}$ and $A_\gamma{}^{Leu}$, giving weak negative CD fine structure (268 and 262 nm) comparable to that found in the denatured Anson material. The positions and intensities of these phenylalanyl CD bands resemble those observed for unoriented phenylalanine side chains[35]. Treatment with guanidine-HCl also diminished the broad positive CD band in all preparations, but the Anson material still retained a much more intense CD between 255 and 270 nm (Fig. 1).

Two additional experiments were performed to verify that the difference between the Anson material and the homogeneous species is not an artifact resulting from minor differences in handling these samples. Since the Anson material originally contained toluene for preservation, the effect of toluene on carboxypeptidase $A_\gamma{}^{Leu}$ was examined. Crystals of carboxypeptidase $A_\gamma{}^{Leu}$ were stored at 5° in water saturated with toluene. After 7 days, these crystals were washed to remove the toluene, just as described for the Anson crystals (see EXPERIMENTAL SECTION), and then dissolved in 25 mM Tris–HCl (pH 7.5) containing 1 M NaCl. The spectrum of this solution showed that prior exposure to toluene does not alter the CD bands of carboxypeptidase $A_\gamma{}^{Leu}$. As a further control, a 12 μM pseudo carboxypeptidase A (Anson) was prepared by mixing 57% carboxypeptidase $A_\gamma{}^{Val}$ and 43% $A_\gamma{}^{Leu}$. This mixture possessed the weak, positive CD (255–270 nm) characteristic of the homogeneous species.

CD spectrum of carboxypeptidase $A_{\alpha+\beta}$ *at 24°*

Fig. 2 shows the near and far ultraviolet CD spectrum of the native carboxy-

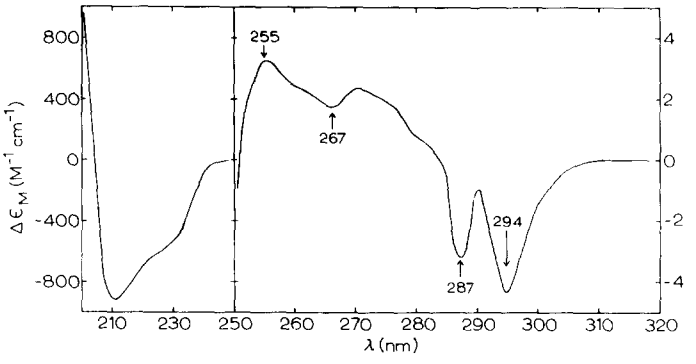

Fig. 2. CD spectrum of carboxypeptidase $A_{\alpha+\beta}$ dissolved in 25 mM Tris–HCl + 0.4 M NaCl (pH 7.5) at 24°. $\Delta\epsilon_M$ is based on the molar concentration of carboxypeptidase A.

peptidase $A_{\alpha+\beta}$. This spectrum is generally similar to the spectra observed for carboxypeptidase A_γ^{Val} and A_γ^{Leu}. The 287- and 294-nm CD bands, however, are about 25% more negative in carboxypeptidase $A_{\alpha+\beta}$ than in carboxypeptidase A_γ^{Val} and A_γ^{Leu}.

Effect of temperature upon the CD spectrum of carboxypeptidase A (Anson)

The initial measurements were made on a frozen solution of carboxypeptidase A (Anson) cooled to −196° (Fig. 3). At this temperature, the negative CD bands previously observed at 294 and 287 nm are greatly intensified, sharpened, and shifted slightly toward shorter wavelengths (293 and 286 nm). Cooling brings out a strong negative CD band at 279 nm and a distinct shoulder at 299 nm. The positive CD band at 262 nm is not much affected by cooling, except in the regions overlapped by the negative CD bands.

An examination of the individual CD bands permits identifying which side

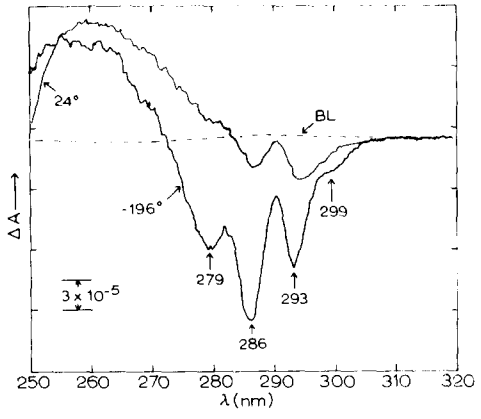

Fig. 3. Instrument trace of CD spectra of 1.1 mM carboxypeptidase A (Anson) in water glycerol with 25 mM Tris–HCl + 0.4 M NaCl (pH 7.5) at 24° and −196°, 0.12-mm path, 20 scans. BL (——) designates water–glycerol baseline. Upon cooling to −196° the area under the CD curve increased 2.8-fold between 290 and 310 nm and 7.3-fold between 282 and 290 nm. The peak-to-peak noise in these instrument traces varies from $1 \cdot 10^{-6}$ M (295 nm) to $6 \cdot 10^{-6}$ M (250 nm).

chains have undergone a conformation change as a result of cooling. The increased intensity of the CD bands at 293 and 299 nm involves tryptophanyl side chains. The negative band at 279 nm is located in a region where either tyrosyl and tryptophanyl fine structure may occur[2]. For example, L-valyl-L-tryptophanyl diketopiperazine at −133° has three fine structure CD bands[4] with about the same spacing as observed in

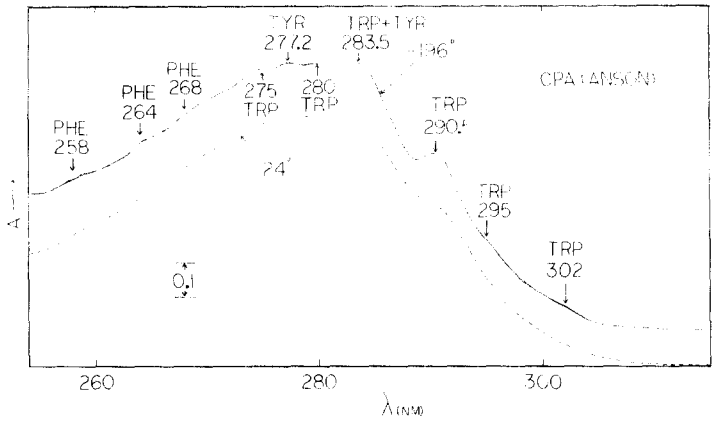

Fig. 4. Absorption spectra of 0.94 mM carboxypeptidase A (Anson) in water–glycerol with 25 mM Tris–HCl + 0.4 M NaCl (pH 7.5) at 24 and −196°. 0.12-mm path. Base line for −196° spectrum was offset 0.1 unit to separate the two spectra. Both solvent base lines were flat.

carboxypeptidase A. In addition, buried tyrosyl residues can have CD fine structure at 279 nm and also at 286 nm[3]. At −196° the 286-nm CD band may arise partly from tryptophanyl side chains and partly from tyrosyl side chains. This possible interpretation is consistent with the observation that the CD intensification upon cooling is greatest in the region where the buried tyrosyl CD bands can occur, *i.e.*, between 270 and 290 nm.

The intensification of the near ultraviolet CD bands occurs without any corresponding increase in absorption intensity. Although cooling to −196° causes some sharpening of absorption bands, the area under the absorption spectrum from 250 to 310 nm changes by less than 10% (Fig. 4). At −196°, the most prominent absorption bands occur at 290.5 and 283.5 nm; weak bands occur at 302, 295, 280, 277.2, 275, 268, 264, and 258 nm. The aromatic side chains giving rise to these bands are identified in Fig. 4, using the information gained from studies of model compounds[1,2,3,36]. The wavelength positions of the bands in the absorption spectrum differ from those observed in the CD spectrum.

Although measurements on water–glycerol glasses at −196° indicate the approximate increase in the CD intensities upon cooling, a more complete investigation can be made using water–glycerol solutions kept above their phase-transition point. Fig. 5 presents the CD spectra of carboxypeptidase A (Anson) at 24, 0, −25, and −40°, where the solutions remained in the liquid state throughout the measurements. The positive CD band at 262 nm is unaffected by cooling. Above 275 nm the CD spectra show a gradual sharpening and a major intensification as the temperature

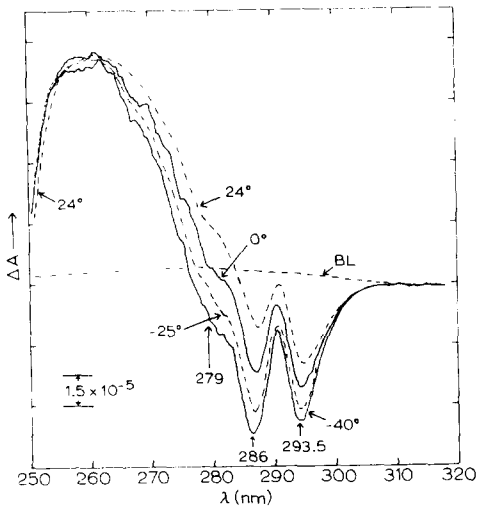

Fig. 5. Variable temperature CD spectra of 12.4 μM carboxypeptidase A (Anson) in water–glycerol solutions containing 0.4 M NaCl and 25 mM Tris–HCl (pH 7.5). Solid lines (−40 and 0°) are actual instrument traces of spectra. The peak-to-peak noise in these records varies from $1 \cdot 10^{-6}$ ΔA (295 nm) to $3 \cdot 10^{-6}$ ΔA (250 nm). At −25 and 24° (— · — · —) the instrument noise has been smoothed out to facilitate distinguishing among the 4 records. 1.0-cm path, 20 scans. BL designates water–glycerol baseline. Upon cooling from 24° to −40°, the area under the CD curve increased 1.6-fold between 290 and 310 nm and 3.8-fold between 283 and 290 nm.

is lowered. By −40° the negative CD band at 279 nm has begun to emerge. The CD bands at 286 and 279 nm intensify more upon cooling than does the tryptophanyl region above 290 nm. These CD changes, which are fully reversed upon rewarming the solutions to 24°, are consistent with the spectra recorded at −196°.

The CD intensification observed upon cooling carboxypeptidase A (Anson)

TABLE I

$\Delta\varepsilon_M$ VALUES AS A FUNCTION OF CARBOXYPEPTIDASE A (ANSON) CONCENTRATION

The enzyme was dissolved in water–glycerol (1:1, by vol.) containing 0.4 M NaCl and 25 mM Tris–HCl (pH 7.5). The path length was varied so that the absorbance remained below 1 and above 0.5 for each concentration.

Concn. (μM)	$\Delta\varepsilon_M$* ($M^{-1} \cdot cm^{-1}$)		$\Delta\varepsilon_M$** ($M^{-1} \cdot cm^{-1}$)	
	24°	−30°	24°	−30°
6	−3.5	5.6	2.3	−5.6
12	3.3	5.6	2.2	−5.9
60	3.4	−5.5	2.3	5.8
140	−3.5	−5.6	−2.3	−5.9
250	3.3	5.6	2.1	−6.0
500	3.4	−5.8	2.3	−6.5
700	3.1	−5.7	−1.9	−6.3
900	3.1	−5.7	−2.1	6.5
1100	−3.2	−6.3	−2.3	−7.3
1250	3.0	−6.0	−1.9	−6.9

* For negative band near 294 nm.
** For negative band near 287 nm.

does not result from aggregation. This conclusion is based upon CD data recorded at carboxypeptidase A concentrations differing by 200-fold (Table I). The $[\theta]_M$ values of the two major negative CD bands do not deviate from Beer's law at either 24 or −30°. Thus the increased CD strength between 275 and 300 nm indicates that cooling alters the orientations of the tryptophanyl and perhaps the tyrosyl side chains of carboxypeptidase A (Anson).

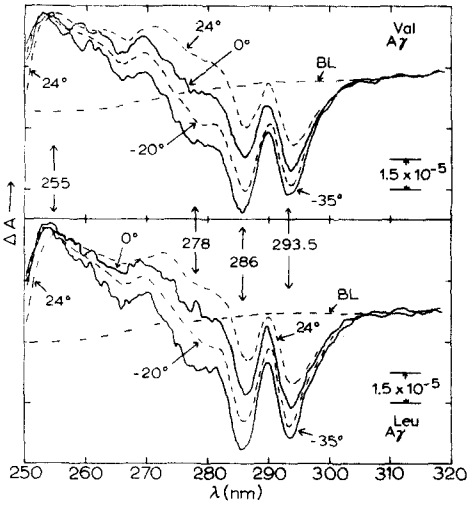

Fig. 6. Variable temperature CD spectra of 51.7 μM carboxypeptidase A_γ^{Val} (Top) and 59 μM carboxypeptidase A_γ^{Leu} (Bottom) in water-glycerol solutions containing 0.4 M NaCl and 25 mM Tris–HCl (pH 7.5). Solid lines (−35° and 0°) are instrument traces of spectra having peak-to-peak noise of $2 \cdot 10^{-6}$ ΔA (295 nm) to $5 \cdot 10^{-6}$ ΔA (250 nm). 2-mm path, 8 scans. BL designates water-glycerol baseline. Similar results were obtained using 12 μM carboxypeptidase A_γ^{Leu} in a 1.0-cm path. Upon cooling from 24° to −35°, the area under these CD curves increased 1.9-fold between 290 and 310 nm and 4.3-fold between 283 and 290 nm.

The conformational changes induced by cooling appear to be confined to the side chain orientations. After taking into account the small solvent contraction, there is no measurable change in the CD spectrum between 250 and 200 nm caused by cooling to either −40 or −196°. Apparently cooling does not cause any major change in the organization of the peptide backbone in carboxypeptidase A (Anson).

Effect of temperature upon the spectra of carboxypeptidase A_γ^{Val} *and* A_γ^{Leu}

Fig. 6 shows that the temperature dependence of the near ultraviolet CD spectra of these two species is essentially identical. Cooling to −35° intensifies the negative CD bands at 294 and 286 nm and brings out a negative shoulder at 278 nm. The emergence of the latter negative band pulls down the CD curve between 260 and 280 nm. The positive CD around 255 nm, however, is not affected by cooling.

In the far ultraviolet, measurements were made using carboxypeptidase A_γ^{Leu}. Cooling to −35° does not alter the CD strength between 200 and 250 nm. In this case also the conformation changes caused by cooling appear limited to the side chains of certain aromatic residues.

Effect of temperature upon the CD spectrum of carboxypeptidase $A_{\alpha+\beta}$

As shown in Fig. 7, cooling carboxypeptidase $A_{\alpha+\beta}$ to $-40°$ affects the near ultraviolet CD spectrum in approximately the same way as described for carboxypeptidase A_γ^{Val} and A_γ^{Leu}. In the region between 200 and 250 nm, cooling carboxypeptidase $A_{\alpha+\beta}$ to $-40°$ does not alter the CD intensity. Measurements on carboxypeptidase $A_{\alpha+\beta}$ at $-196°$ revealed negative CD bands at 299, 293, 285.5, and 279 nm. In addition, weak negative phenylalanyl CD fine structure was evident at 261 and 268 nm.

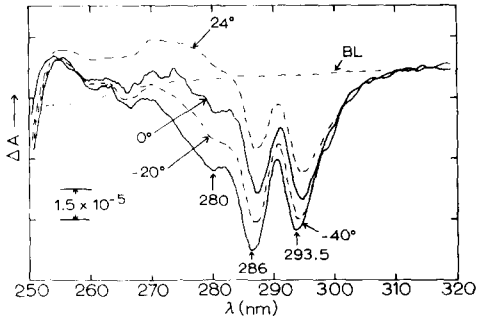

Fig. 7. Variable temperature CD spectra of 12 μM carboxypeptidase $A_{\alpha+\beta}$ in water glycerol solutions containing 0.4 M NaCl and 25 mM Tris-HCl (pH 7.5). Solid lines (−40 and 0°) are instrument traces of spectra having peak-to-peak noise of $2 \cdot 10^{-6}$ ΔA (295 nm) to $5 \cdot 10^{-6}$ ΔA (250 nm). 1.0-cm path, 8 scans. Upon cooling from 24° to −40°, the area under the CD curve increased 1.5-fold between 290 and 310 nm and 3.1-fold between 283 and 290 nm.

Validation of certain experimental procedures

Irradiation

Since there have been reports that ultraviolet irradiation causes inactivation of carboxypeptidase A and selective destruction of its tryptophanyl and tyrosyl residues[37-39], the effect of prolonged scanning in our CD spectrophotometer was examined. A 12 μM solution of carboxypeptidase A (Anson) in 0.4 M NaCl 25 mM Tris-HCl (pH 7.5) was irradiated at 24° (scanning from 320 to 245 nm) in the CD instrument using a normal slit width (0.6 nm). Even after 40 scans (3.2 h) there was no significant difference between the peptidase activity of the irradiated carboxypeptidase A (Anson) sample and that of the unirradiated enzyme solution. Another test for possible radiation damage involved comparing the CD spectra after averaging different numbers of scans. This comparison, using the various carboxypeptidase A preparations, confirmed that the CD intensities were unaltered during repeated scanning; only the noise diminished. Thus our CD measurements did not cause any measurable damage to these enzymes.

Glycerol

The possible effects of 50% glycerol on carboxypeptidase A were examined. The peptidase activities of carboxypeptidase A (Anson), A_γ^{Val}, A_γ^{Leu}, and $A_{\alpha+\beta}$ were unaltered when water–glycerol (1:1, by vol.) was used in place of the water solvent in the assay procedure (Table II). Furthermore, 50% glycerol did not change the CD spectra of the various preparations of carboxypeptidase A at 24°. Apparently the

TABLE II

PEPTIDASE ACTIVITY OF THE VARIOUS CARBOXYPEPTIDASE A PREPARATIONS AT 24°

All assays were done at pH 7.5 using 1 mM *N*-benzoyl-Gly-L-Phe and $1 \cdot 10^{-8}$–$2 \cdot 10^{-8}$ M enzyme.

Solvent	*Turnover number* (min^{-1})*			
	Anson	A_γ^{Leu}	A_γ^{Val}	$A_{\alpha+\beta}$
0.4 M NaCl–25 mM Tris in water	1850	1920	1830	2100
1.0 M NaCl–25 mM Tris in water	2100	2150	2200	—
0.4 M NaCl–25 mM Tris in water–glycerol (1:1, by vol.)	1950	1850	1900	2100

* Each value is the average of at least 3 trials.

water–glycerol solvent does not alter the conformation of any of these carboxypeptidases.

Freezing

The possible effects of freezing carboxypeptidase A (Anson) to −196° in water–glycerol (1:1, by vol) with 0.4 M NaCl–0.025 M Tris (pH 7.5) were also studied. The peptidase activity assayed at 24° was the same before and after freezing the enzyme. In addition, the near ultraviolet CD spectral changes caused by freezing were fully reversed upon rewarming the carboxypeptidase A (Anson) to room temperature. Apparently freezing under our experimental conditions did not damage carboxypeptidase A.

Effect of pH

Measurements showed that the pH of the water-glycerol–25 mM Tris–0.4 M NaCl solutions (pH 7.5) at 24° increased about 0.3 units upon cooling to 0°. An additional increase in pH may have occurred at lower temperatures. Consequently the CD spectrum of carboxypeptidase A (Anson) was also recorded at other pH's (8.6, 8.4, 8.2, 7.9, and 6.7). The intensities of the CD bands at 294 and 287 nm remained constant within this pH range. Evidently the CD intensification upon cooling cannot be due to pH changes which may occur in the cold tris buffer.

DISCUSSION

The CD spectra of the various carboxypeptidase A species can be used to compare their conformations. The purified carboxypeptidase A_γ^{Val}, A_γ^{Leu}, and $A_{\alpha+\beta}$ species have nearly identical CD spectra in both the near and far ultraviolet region (Figs. 1 and 2). The CD findings in the 200 to 250-nm region suggest that the backbone conformations of these species are similar. Although carboxypeptidase A_α has 7 more residues (Ala–Arg–Ser–Thr–Asn–Thr–Phe) than A_γ at the N-terminus[13], the CD spectra are not sufficiently precise to detect these few additional residues in a protein containing 300 residues. The similarity of the CD intensities from 280 to 300 nm indicates that the environments of the tryptophanyl side chains are also nearly identical in the different species.

The CD spectra permit comparing carboxypeptidase A at different stages of purification (Fig. 1). PÉTRA AND NEURATH[15,40] have reported isolating carboxypeptidase A_γ^{Val} (40%), A_γ^{Leu} (30%), A_β^{Val} (15%), A_β^{Leu} (10%), and A_α (2%) from

a commercial A (Anson) preparation. Thus it is noteworthy that the Anson material has a much more intense positive CD band (255–270 nm) than does carboxypeptidase A_γ^{Val}, A_γ^{Leu}, or $A_{\alpha+\beta}$, the major components of the Anson preparation.

In principle, the lower CD intensity (255–270 nm) of the purified carboxypeptidase A_γ species might be explained by any of the following: (A) an impurity which is removed from the Anson material during purification, (B) a subtle conformation change occurring during purification, or (C) an interaction among the various species of carboxypeptidase A in the Anson preparation. The latter possibility appears remote because a mixture of carboxypeptidase A_γ^{Val} and A_γ^{Leu} had the low CD intensity characteristic of the purified species between 255 and 270 nm.

The only protein impurity known to occur in the Anson enzyme is the breakthrough peak, which apparently comprises less than 5% of the total protein[15]. If this protein were to account for the loss of CD intensity during purification, its $l\varepsilon_M$ would have to be at least 100 $M^{-1} \cdot cm^{-1}$ at 262 nm. Such a large value seems improbable. Another possible impurity is toluene, which is present in the Anson material before the first wash. Control experiments, however, showed that toluene is not responsible, since prior exposure to toluene did not affect the CD intensity of carboxypeptidase A_γ^{Leu}.

The possibility of a conformation change during preparation of the purified carboxypeptidase A_γ species is not without precedent. Apparently both carboxypeptidase A_δ and A_γ have identical amino acid sequences and the same number of residues[13]. Nevertheless, their solubilities differ appreciably, which suggests that their conformations are not identical.

If a conformation change does occur during purification of carboxypeptidase A (Anson), then the CD spectra (Fig. 1) suggest that the alteration involves the environments of some phenylalanyl side chains[1,35] and possibly also of the disulfide bond[31–34]. The difference in the phenylalanyl CD bands (268–262 nm) was eliminated after treating the various preparations with guanidine hydrochloride to disorder the phenylalanyl side chains. In contrast, guanidine hydrochloride did not completely abolish the difference in the positive CD band (255–270 nm) between the Anson material and the purified A_γ species (Fig. 1). This observation seems consistent with identifying the large positive CD band (255–270 nm) as arising mainly from the disulfide bond. This covalent link between two parts of the peptide chain may prevent guanidine·HCl from thoroughly disrupting the ordered side chains vicinal to the disulfide bond. The relatively constant specific activity of the various preparations[15] does not preclude a difference in the environment or dihedral angle of the disulfide bond, since an intact disulfide bond is not required for enzymic activity[29] of carboxypeptidase A.

Next we shall consider the effects of temperature upon the conformations of carboxypeptidase A. The observed CD spectra represent the population-weighted average from all conformers of carboxypeptidase A existing in equilibrium at each temperature[41,42]. MOSCOWITZ *et al.*[41] have described the effect of temperature upon the CD spectra of a simplified system of two conformers in which the difference in Gibbs free energy between conformers is independent of temperature. Cooling such a system causes a shift to the lower energy state in accordance with the Boltzmann factor ($\exp(-\Delta G/RT)$). Unfortunately, in the case of proteins, more than two conformers of each amino acid residue may be in equilibria; and, furthermore, the

energy difference between any two conformers probably varies with temperature. For example, the Gibbs free energy change for hydrophobic interactions involving aromatic side chains becomes more positive at lower temperatures[43]. Upon cooling a protein the conformational equilibria are shifted by the reduction in RT and also by any changes that may occur in the Gibbs free energies separating the various conformers. Although these difficulties preclude a quantitative evaluation of the conformer populations in a protein, a qualitative description is possible.

The variable temperature CD spectra of the carboxypeptidase A's give information about the extent of motion in these proteins. Since the far ultraviolet CD bands of carboxypeptidase A are not altered by cooling, the peptide backbone conformation appears to be relatively rigid. In contrast, cooling causes a major increase in the rotatory strength of the CD bands arising from the tryptophanyl and perhaps from the tyrosyl side chains (Fig. 3). For example, the area under the CD band at 293 nm increases at lower temperatures in much the same way as has been observed in the CD spectra of tryptophanyl diketopiperazines[4]. This result suggests that at room temperature one or more tryptophanyl side chains of carboxypeptidase A have multiple conformers, which interconvert by rotations about single bonds, *i.e.* conformational equilibria[2,41,42]. In the biological literature this condition has been called conformational motility[12]. Cooling carboxypeptidase A reduces the motility of the tryptophanyl side chains and thereby increases the total rotatory strength of their CD bands[2].

This qualitative description appears correct even if the relative free energies of the various conformers do change upon cooling. The continued increase in CD strength as the temperature is lowered (Figs. 5–7) suggests that the rearrangements of the tryptophanyl side chains result from noncooperation interactions[44]. The alternative possibility, a cooperative interaction involving many amino acid residues moving simultaneously, would be expected to show a sharp transition temperature[44], which is not observed.

The motility of the tryptophanyl side chains observed for carboxypeptidase A in solution agrees qualitatively with the conclusions drawn from X-ray data on crystals. If a side chain has several conformers in a crystalline protein, then the electron density of the side chain will appear somewhat diffuse. The X-ray data obtained by LIPSCOMB and co-workers[9,10] were used to determine the disorder of the tryptophanyl side chains in crystalline carboxypeptidase A_α (Table III). The side chains of tryptophan residues 126, 257 and 294 have appreciable disorder in the crystal. These side chains may contribute to the tryptophanyl CD bands. The indolyl rings of tryptophans 126, 257 and 294 are sufficiently close to other aromatic rings (Table III) that strong dipole–dipole coupling probably gives rise to appreciable CD strength in carboxypeptidase A, just as has been found in model compounds[4]. Other interactions may also contribute to the tryptophanyl CD bands[45,46]. In any case, the CD bands induced by these interactions would be intensified as the relative motion between the indolyl rings and their surroundings is reduced by cooling. Since it is the relative motion of groups that influences the CD intensity, the observed increase in tryptophanyl CD strength may also involve decreased motion in the moieties which interact with the indolyl ring. Finally, it should be noted that the other 4 or 5 tryptophan residues[47–49] may also contribute to the CD bands of carboxypeptidase A.

TABLE III

CHARACTERISTICS OF INDOLYL RINGS IN CARBOXYPEPTIDASE A_α

Based upon observations by L. F. using the molecular model and electron density maps in Prof. Lipscomb's laboratory.

Trp No.	*Environment of indolyl ring*	*Relative disorder* in crystal at 4°*	*Neighboring aromatic residues***
63	Buried in hydrophobic pocket	None	Phe 192 (5.2 Å), Phe 189 (6.2 Å), Phe 182 (7.3 Å)
73	6-membered ring partially exposed, 5-membered ring in hydrophobic pocket	Slight	Tyr 12 (5.3 Å), Phe 116 (5.9 Å)
81	Exposed to solvent	None	Tyr 9 (6.7 Å), Phe 82 (7.0 Å) Trp 294 (7.3 Å)
126	Exposed to solvent	Moderate	Phe 118 (5.3 Å)
147	Buried in hydrophobic pocket	Slight	Trp 257 (6.5 Å), Phe 192 (6.7 Å)
257	Mostly exposed	Heavy	Trp 147 (6.5 Å)
294	6-membered ring exposed, 5-membered ring in hydrophobic pocket	Heavy	Phe 82 (6.2 Å), Phe 86 (6.6 Å), Phe 96 (6.6 Å), Trp 81 (7.3 Å)

* By using the large Mylar electron density maps[10] the relative disorder in the aromatic side chains was assessed from the extent to which ring contours strayed from the computed, best-fit atomic positions labeled on these maps. These "stray" ring contours could often be accounted for by visualizing a rotation of the ring atoms about the $C\alpha$-$C\beta$ or $C\beta$-$C\gamma$ bonds.

** Distances between centers of rings are given in parentheses. Values were computed from refined coordinates supplied by Lipscomb and Reeke (personal communication).

Although the tryptophanyl side chains appear to have multiple conformers in both crystals and solutions of carboxypeptidase A, the extent of motility may not be identical in the two states. From a comparison of enzymic activity in various solid states and in solution, QUIOCHO AND RICHARDS[8] suggested that carboxypeptidase A may have more flexibility in solution than in the crystal. Unfortunately the motility implied by our CD measurements cannot be quantitatively compared with the disorder observed for some side chains in crystalline carboxypeptidase A. Even though side chain motility may be essential to catalysis by enzymes, the motility in the tryptophanyl side chains of carboxypeptidase A does not have a direct bearing on this question, since none of these indolyl rings are located in the active site[9,10]. Furthermore, substrate binding by carboxypeptidase A causes different changes in the CD spectra than does cooling[50].

ACKNOWLEDGMENTS

We are very grateful to Professor Philip H. Pétra and Professor Hans Neurath for kindly supplying the purified fractions of carboxypeptidase A, for reading the manuscript, and for giving a number of helpful comments. We thank Professor William N. Lipscomb and Dr. George N. Reeke, Jr., for several informative discussions, for providing refined coordinates of carboxypeptidase A, and for permitting L. F. to examine their electron density maps and molecular model. We are indebted to Miss Amelia Adams for photographing portions of the carboxypeptidase model. This work was supported by Contract AT (04-1) GEN-12 between the Atomic Energy Commission and the University of California.

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