FUNCTIONAL AND CONFORMATIONAL PROPERTIES OF CARBOXYPEPTIDASE A*†

BERT L. VALLEE

The Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School
and
Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts, USA

ABSTRACT

The conformations of carboxypeptidase $A_{\alpha,\gamma}$ have been investigated by comparing chemical, spectral and kinetic data in solution and crystals with the results of x-ray structural analysis of the enzyme crystals. The data indicate that the conformation(s) in the two physical states differ and the consequent limitations of mechanistic interpretations of structural analysis are discussed. The chemical, spectral and kinetic results show that the physical state of an enzyme is a significant experimental variable that likely induces changes in function-related-conformation. Since the kinetic properties of carboxypeptidase crystals differ markedly from those of solutions, enzyme substrate model building, based on kinetics in solutions, would seem of questionable value in solving mechanistic problems.

INTRODUCTION

Three-dimensional structures of enzymes derived from x-ray analysis of crystals have served increasingly to deduce their mechanisms of action in solution, based on the hypothesis that the conformations in the two physical states are the same. This assumption is critical in assigning functional significance to the structural details of active sites of enzymes. It would be difficult to define the precise roles of amino-acid sidechains thought to be involved in catalytic mechanisms if their positions were to be a function of the physical states of a protein. That proteins can assume multiple and readily interconvertible but closely related conformations has long been suspected[1] and is supported now by x-ray studies of proteins and enzymes indicating that sidechains and segments of peptide chains may be motile[2–5]. Since protein structure analysis is often carried out under con-

* This research was supported by Grant-in-Aid GM-15003 from the National Institutes of Health, of the Department of Health, Education and Welfare.

† Since presentation of this material and submission of the manuscript a more complete version of the relevant data has been published (J. T. Johansen and B. L. Vallee, *Biochemistry*, **14**, 649 (1975).)

ditions that differ from those used for examining function, the documentation of such conformational changes as well as the judgement of their significance, have presented difficulties. The increasing importance of x-ray crystallography in studies of structure–function relationships of enzymes has generated numerous questions concerning possible effects of crystallization on conformation. As yet, no satisfactory answers exist in spite of the fact that knowledge relating to this problem is beginning to accumulate[6]. Thus, the integration of functional data obtained in solution with the structural details derived from crystals remains one of the important problems in discerning the mechanism of action of an enzyme.

The properties of carboxypeptidase have been studied in particular detail from this point of view. The data show that the specific activity of carboxypeptidase in the crystalline state is only about 0.3 per cent of that in solution when examined with the substrate carbobenzoxyglycyl-L-phenylalanine[7–9]. The two physical states also differ in the case of zinc removal, that of inhibition, and in the enzymatic consequences of inorganic and organic modifications, though the basis of these phenomena has remained obscure. We have therefore searched for probe characteristics that could simultaneously assess activity and essential structural features of the active centre while being dynamically responsive to a wide range of environmental conditions[10].

In these studies chromophoric probes have been particularly helpful. Both diazonium salts and tetranitromethane (TNM) have proved valuable for introducing environmentally sensitive chromophores into proteins to signal local conformational changes which may reflect functional properties[10–13]. Among the diazonium salts, diazotized *p*-arsanilic acid has proved exceptionally important for such studies of carboxypeptidase A. In crystals of the enzyme, this reagent specifically labels Tyr-248[14–17]. The resulting azotyrosyl-248 chromophore monitors local conformational changes as a function of the physical state in which the enzyme finds itself[15, 16].

Such azophenols form complexes with zinc and other metals[18]; concomitantly their colour changes from yellow to red or orange. The extensive information available on the composition, structure and function of carboxypeptidase A[19–21] makes this enzyme particularly suitable to examine the proximity of such an azophenol to the zinc atom by this means.

Chemical studies have shown that the zinc atom and tyrosyl residues of the enzyme are essential to peptide hydrolysis[20]. Moreover, based on x-ray analysis, a critical catalytic role has been assigned to Tyr-248; it is thought to donate a proton to the scissile peptide bond of the substrate[19]. The location of Tyr-248 with respect to the active-site Zn atom has been thought decisive in relating the structure of the particular crystals of carboxypeptidase A_α used for x-ray analysis to the catalytic mechanism of carboxypeptidase in general[22, 23]. These studies place Tyr-248 at the molecule surface, with its sidechain pointing away from the active site and 17 Å from the Zn atom.

SCOPE OF PRESENT WORK

In solution, the distance between Tyr-248 and the Zn atom is unknown. and direct, quantitative methods for such measurements have not as yet been devised. However, the introduction of a suitable probe and its interaction

with zinc might result in the formation of an intramolecular coordination complex. Its existence or absence might then qualitatively signal the proximity of these two components of the active centre.

In our hands, crystals of carboxypeptidase A_α, A_β and A_γ, when specifically coupled with diazotized arsanilic acid are all yellow, but their solutions are red with an absorption maximum at 510 nm, characteristic of an intramolecular coordination complex between mono-arsanilazotyrosine-248 and Zn†. This complex can be dissociated and its accompanying absorption and CD spectra can be abolished by crystallization of the enzyme, removal of the metal, substrates, inhibitors or denaturation. In turn, it can be restored by dissolving crystals, adding zinc to the azoapoenzyme, by removal of substrates and inhibitors[15, 16]. The pH dependence of this spectrum provides detailed information regarding the molecular basis of its origin, demonstrating that at pH 8.2 the participating species indeed include the azophenolato and azophenolate species of the enzyme and its zinc atom. The complex forms in the solution but not in the crystalline state.

This indicates that in solution under these conditions Tyr-248 is close enough to the active site zinc atom to form an intramolecular coordination complex, i.e. it is much closer than was indicated by x-ray studies of crystals[19] where this complex does not form[15, 16].

A number of other approaches have all confirmed this conclusion. Thus, the conformation of the crystals and solutions of nitrotyrosyl-248 carboxypeptidase also differs[24]. This makes it quite unlikely that introduction of either the azo- or the nitro-probe is responsible for the local, structural differences.

Phosphorescence studies of native CPD B have given further evidence that a tyrosyl residue is close to the zinc atom of that enzyme[25].

Finally, detailed kinetic studies both of the solutions and crystals of native CPA A_α and A_γ and the determination of their respective kinetic parameters provide most convincing evidence that the conformations of the two physical states differ, regardless of the particular isomeric species of the enzyme examined[26, 27].

SPECTROPHOTOMETRIC–pH TITRATIONS

The following will present some of the details which form the basis of these conclusions.

The spectra and activities of the α, β and γ zinc azoenzymes are identical. The crystals are yellow but turn red when dissolved at pH 8.2, as would be expected when azoTyr-248 and zinc form a complex. Spectral titration [*Figure 1*(a)] of zinc azocarboxypeptidase generates an absorption maximum

† To simplify nomenclature, zinc carboxypeptidase A (the zinc azoenzyme) and apoazocarboxypeptidase A (the apoazoenzyme) are used interchangeably with zinc monoarsanilazotyrosine-248 carboxypeptidase and apoarsanilazotyrosine-248 carboxypeptidase, respectively, of any enzyme form. Carboxypeptidase A_α used for x-ray structure analysis and with a crystal habit elongated along the *a* axis[23] is designated the x-ray crystal or enzyme. Azo-Tyr-248 refers to mono-arsanilazotyrosine-248, the azophenolate ion to its ionized species. The absorption spectrum of zinc monoarsanilazotyrosine-248 carboxypeptidase is defined as 'yellow' in the absence of an absorption band at λ_{max} 510 nm, as 'red' in its presence.

at 510 nm (ε = 8000)‡ between pH 6.3 and 8.5. On raising the pH to 10.8, the maximum shifts progressively to 485 nm (ε = 10500), typical for the free azophenolate ion. Over the pH range from 6.3 to 8.5 there is one isosbestic point at 428 nm, replaced by two isosbestic points between pH 8.5 and 10.8, now at 412 and 520 nm, direct evidence for the progressive formation of at least three interconvertible species, i.e. the azophenol, the complex and the azophenolate ion.

The spectral response to titration of the apoenzyme over the same pH range is completely different [*Figure 1*(b)]. The 510 nm absorption band does not appear, as would be expected in the absence of zinc, and only that

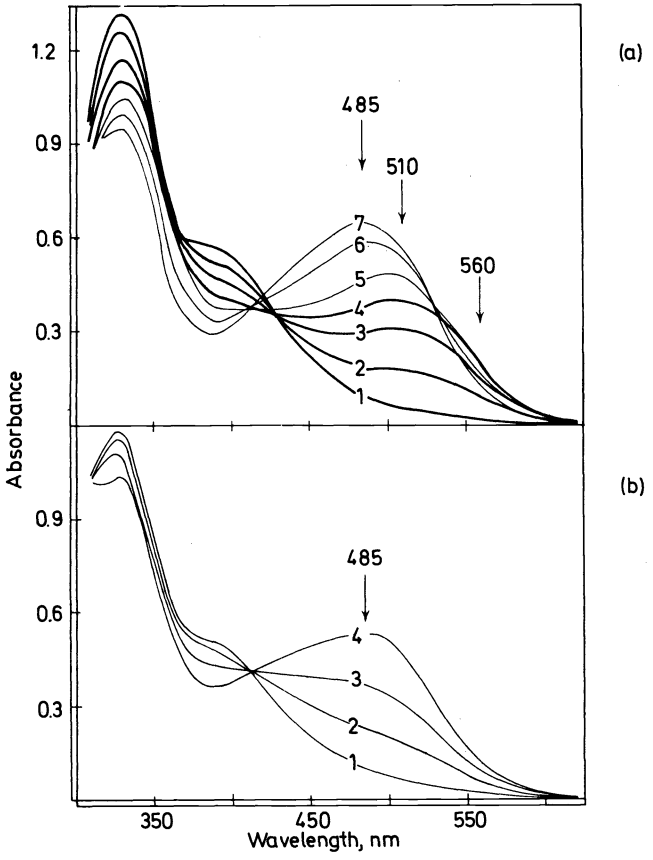

Figure 1. Effect of pH on the absorption spectrum of (a) zinc arsanilazotyr-248 carboxypeptidase$_{\alpha,\beta,\gamma}$ and (b) apoarsanilazotyr-248 carboxypeptidase$_{\alpha}$ both in 2 mM Tris·HCl–0.5 M NaCl. The numbers indicate the pH at which the spectra were recorded: (a) (1) pH 6.2; (2) pH 7.3; (3) pH 7.9; (4) pH 8.3; (5) pH 8.8; (6) pH 9.6; (7) pH 10.8. (b) (1) pH 7.5; (2) pH 8.7; (3) pH 9.3; (4) pH 9.9. At higher pH values, the apoenzyme denatures. The arrows in this and succeeding figures identify maximal absorbances (or differences).

‡ The absorbance and molar ellipticity values for the azoTyr-248·Zn complex are given at pH 8.5 and 23° ± 0.1, those for the azophenolate ion at pH 10.8 and 23° ± 0.1.

of the azophenolate ion at 485 nm is generated[15, 16] and, significantly, only after the pH is increased above pH 8.5. A plot of absorbance at 485 nm versus pH reveals a pK of 9.4 [*Figure 2*(c)], characteristic of the ionization of the arsanilazophenol and identical to that of mono-arsanilazo *N*-acetyl and *N*-carbobenzoxytyrosine and other similar mono-arsanilazotyrosine derivatives[28].

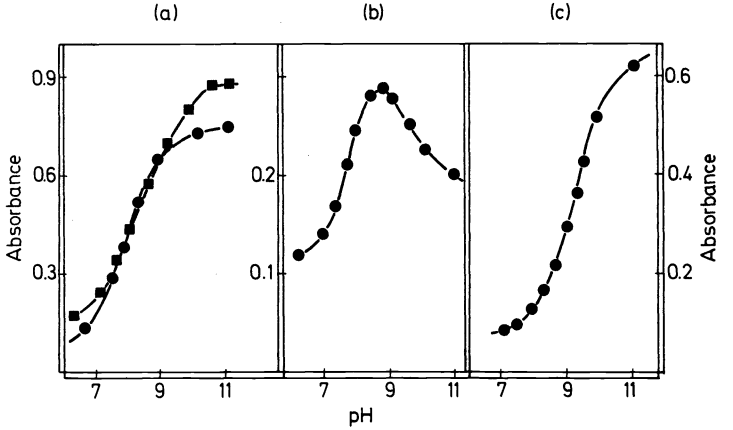

Figure 2. Absorbance–pH titration from the spectra in *Figure 1*(a) and (b) of (a) zinc arsanilazotyr-249 carboxypeptidase$_{\alpha,\beta,\gamma}$ at 485, 510 and (b) 560 nm, respectively, and of (c) apoarsanilazotyr-248 carboxypeptidase$_{\alpha}$ at 485 nm. The value at pH 11 is calculated.

Figure 2(a) shows the pH titration curves of the zinc azoenzyme at 485 and 510 nm and that at 560 nm [*Figure 2*(b)] the wavelength where absorbance of complex and azophenolate ion differ maximally [*Figure 1*(a)]. Neither at 485 nm nor at 510 nm does the titration fit a theoretical titration curve for a single pK when examined over a wide pH range. Furthermore, that performed at 560 nm reveals two pK values at 7.7 and 9.5, respectively, and fits a theoretical curve consistent with the existence of a metal complex.

Lipscomb and co-workers have reported a pH titration curve of the enzyme used for x-ray analysis at one wavelength only, 510 nm, and over a narrow pH range. It was interpreted to indicate the existence of only a single ionization, pK_a 7.78 $\pm$ 0.04, for zinc azocarboxypeptidase A_α and pK_a 7.72 $\pm$ 0.64 for the modified γ enzyme. These pH titrations were apparently insensitive to the substantial contribution of the azophenolate species to the 510 nm absorbance [*Figure 2*(a)]. They were performed at very low absorbance and were not extended beyond pH 9, where the spectral characteristics of the azophenolate ion would have become obvious [*Figure 2*(a)]. These titrations are incomplete and do not permit the mechanistic deductions which were based on these data[23].

Circular dichroic measurements can vary not only in amplitude but also in sign. In the present instance they resolve the spectral contribution of the complex from that of the azophenolate ion [*Figure* 3(a)]. Increasing pH from 6 to 8.5 generates the negative ellipticity band at 510 nm, specific for

the azotyr-248 Zn complex. At pH 8.5 the value of the negative extremum at 510 nm is maximal, $[\theta]^{23^\circ} = -43000$. At higher pH values a positive band for the azophenolate ion is found at 485 nm, $[\theta]^{23^\circ} = +10500$. Though the ellipticity maxima of these two species are separated by only 25 nm, circular dichroism [*Figure 3*(a)] of the zinc azoenzyme can distinguish these unambiguously owing to their difference in sign. In accordance with this, the apoenzyme only exhibits the positive rotation at 485 nm associated with the azophenolate species. The apoenzyme completely lacks the large negative ellipticity band at 510 nm over the entire pH range [*Figure 3*(b)], demonstrating the dependence of this negative ellipticity band on the formation of the zinc complex.

The bell-shaped circular dichroism titration curve for the zinc azoenzyme at 510 nm (*Figure 4*) fits a theoretical curve for the ionization of two groups

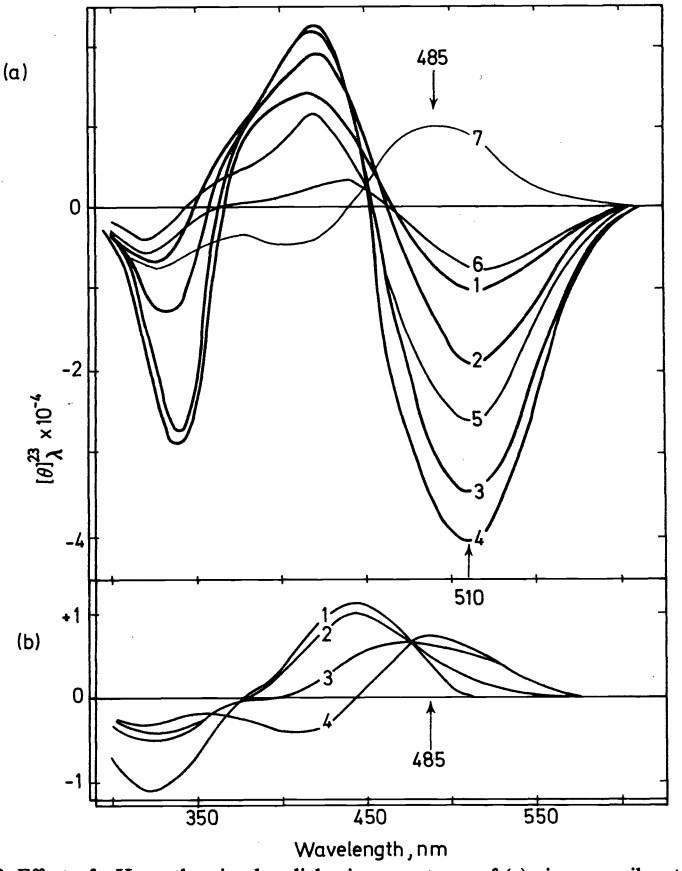

Figure 3. Effect of pH on the circular dichroism spectrum of (a) zinc arsanilazotyrosine-248 carboxypeptidase$_{\alpha,\beta,\gamma}$ and (b) apoarsanilazotyrosine-248 carboxypeptidase$_{\alpha}$ in 0.2 mM Tris·HCl–0.5 M NaCl. The numbers indicate the pH at which the spectra were recorded: (a) (1) pH 6.9; (2) pH 7.3; (3) pH 7.9; (4) pH 8.3; (5) pH 9.4; (6) pH 9.8; (7) pH 10.8. (b) (1) pH 7.2; (2) pH 8.4; (3) pH 9.0; (4) pH 9.9.

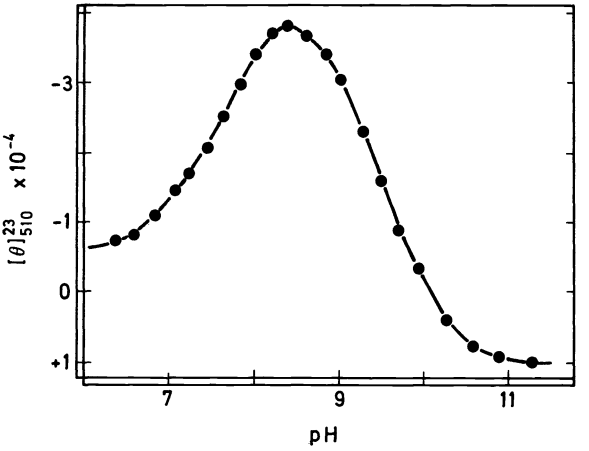

Figure 4. Circular dichroism–pH titration from the spectra in *Figure 3* of zinc arsanilazotyrosine-248 carboxypeptidase at 510 nm in 2 mM Tris·HCl–0.5 M NaCl.

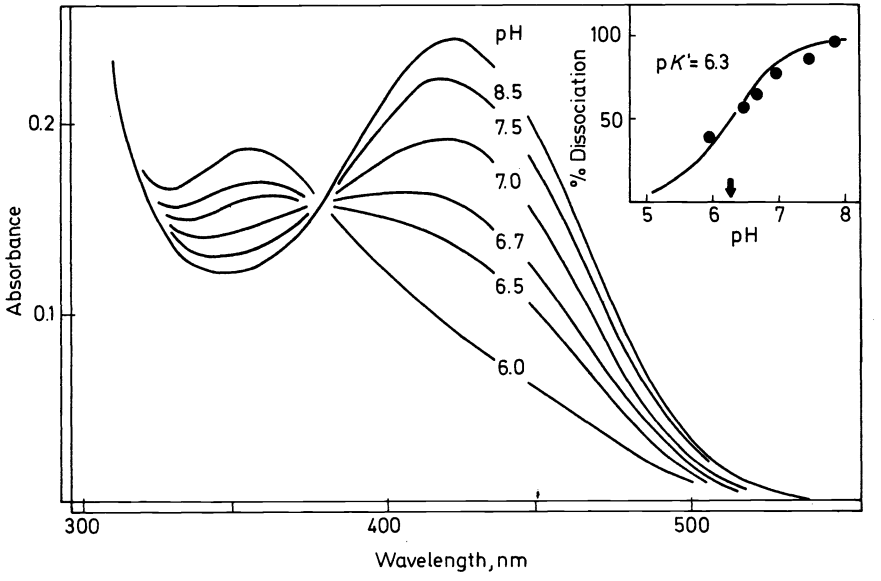

Figure 5. Absorption spectra of a solution of nitrocarboxypeptidase A in 0.002 M Tris–acetate–0.5 M NaCl at the pH values indicated. Inset: Degree of dissociation as a function of pH. The solid line describes a normal ionization curve with a mid-point at pH 6.3 (arrow).

having pK_a values, 7.7 and 9.5, identical to those obtained from the absorbance–pH titration at 560 nm [*Figure 2*(b)]. Moreover, addition of cobalt, nickel, cadmium or manganese to the apoenzyme results in a series of different metallocarboxypeptidases with absorption and circular dichroic extrema characteristic for each metal. Their titrations reveal sets of two pK_a values typical of each particular metal[29].

The results of spectrophotometric pH titrations of monotetraazolyl-*N*-carbobenzoxytyrosine and its Zn complex [*Figures 5* and *6*] resemble those of the apo- and zinc azoenzymes, respectively, to a remarkable degree [*Figures 1*(a) and (b)]. With increasing pH the maximum of the complex at 512 nm ($\varepsilon = 8700$) shifts to 482 nm ($\varepsilon = 8700$) until the spectrum becomes identical to that of the azophenolate ion. A plot of absorbance at 560 nm against pH is bell-shaped, reflecting the formation and dissociation of the complex [*Figure 5*, insert].

The series of chemical events that give rise to these spectral data include the displacement of the azophenolic hydroxyl group proton to form the zinc complex and, at a higher pH, dissociation of the complex to give the azophenolate ion. We conclude, therefore, that the formation of a coordination complex between arsanilazotyr-248 and zinc accounts for the characteristic 510 nm absorption and circular dichroic bands of arsanilazocarboxypeptidase.

Interpretations of the electron density maps have consistently excluded the possibility that Tyr-248 might be able to interact with the zinc atom of the enzyme. In particular, the fact that even in the presence of substrate the OH group of Tyr-248 is still some 4–5 Å away from the zinc atom has apparently presented a major obstacle to the acceptance of the evidence from solution spectra of arsanilazo-Tyr-248-carboxypeptidase that Tyr-248 interacts with zinc. This reluctance can be attributed to the fact, in part, that it was thought to require major re-positioning of the extended polypeptide chain around Tyr-248 to bring about such an interaction in the native enzyme which was deemed unlikely[23]. The x-ray maps of the native enzyme, first published in 1968 in support of the non-existence of the Tyr-248·Zn interaction[19], have now been re-interpreted subsequent to the publication of the above H^+ ion titrations[16] demonstrating formation of the complex azotyr-248·Zn carboxypeptidase spectra. The very same maps are now thought to support the interaction of Tyr-248 with Zn at pH 7.4 in 15–25 per cent of the molecules[30], a proportion of molecules consistent with that predicted by our titration curves of the arsanilazoenzyme analysed on the basis of the intramolecular azotyrosine-248·Zn complex[16]. Further, model building has now uncovered that the conformational change in the polypeptide backbone near Tyr-248 is readily feasible after all, such that the penolate group of Tyr-248 can bind to Zn[30]. Thus, the 'substrate induced' inward movement of Tyr-248 considered to be a direct experimental verification of the 'induced fit' theory[19, 30] has become progressively less tenable. If the interaction of Tyr-248 and Zn exists in the absence of substrate, one must ask why the presence of substrate should be considered essential to bring about the movement of this residue.

Studies of nitrocarboxypeptidase are consistent with such conclusions. Nitration of carboxypeptidase A with tetranitromethane (TNM) increases its esterase activity to 180 per cent and decreases its peptidase activity to about 20 per cent, relative to that of the native enzyme when asayed under standard conditions[11]. These functional changes occur concomitantly with the formation of one nitrotyrosyl residue per molecule of enzyme. At least 80 per cent of the modification occurs at Tyr-248[31]. At pH 6, in solution, nitrocarboxypeptidase exhibits a visible absorption spectrum with a maximum at 360 nm.

On increasing the pH to 8.5, this band decreases and is replaced by another at 428 nm (*Figure 5*), with an isosbestic point at 380 nm. This titration behaviour is typical of nitrophenol ionization. A plot of absorbance at 428 nm versus pH (*Figure 5* inset) follows a titration curve with an apparent p*K* of 6.3, a value distinctly lower than that observed for 3-nitrotyrosine, *N*-acetyl-3-nitrotyrosine or *O*-nitrophenol, 6.8, 7.0 and 7.2, respectively (*Table 1*).

Table 1. p*K'* values for phenolic ionization in various nitrophenols and nitrotyrosyl residues in copolymers*

Substance	p*K'*	Substance	p*K'*
o-Nitrophenol	7.2	Glu-Tyr (9:1)	7.1
3-Nitrotyrosine	6.8	Glu-Lys-Tyr (54:40:6)	6.7
N-Acetyl-3-nitrotyrosine	7.0	Lys-Tyr (10:1)	6.2

* All p*K*'s were estimated from spectrophotometric–pH titration data obtained with solutions of the various substances in 0.2 M tris-acetate-0.5 M sodium chloride.

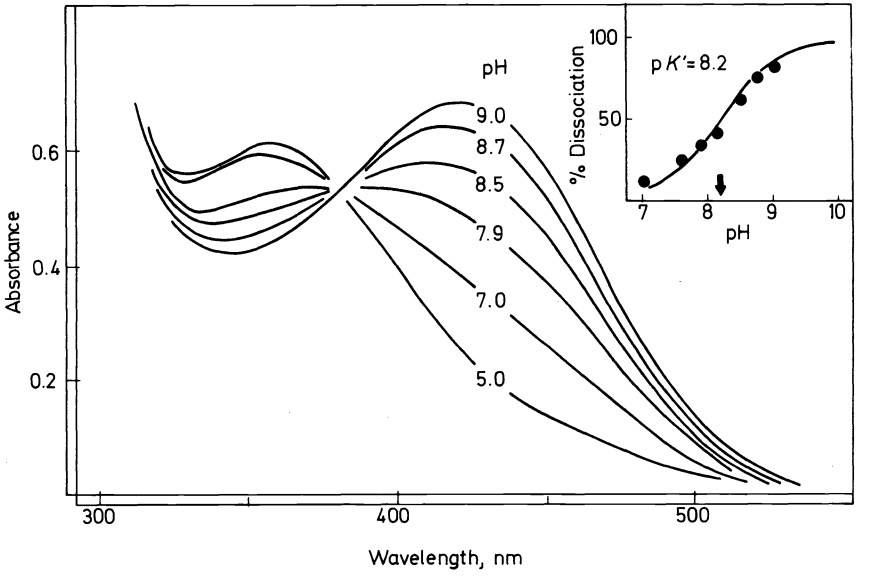

Figure 6. Absorption spectra of a crystal suspension of nitrocarboxypeptidase A in 0.002 M Tris–acetate at the pH values indicated. Inset: Degree of dissociation as a function of pH. The solid line describes normal ionization curve with a mid-point at pH 8.2 (arrow).

Charge effects in the local environment of the nitrotyrosyl residue of carboxypeptidase could account for the decrease in apparent p*K*. Spectrophotometric titrations of a series of nitrotyrosyl-containing copolymers reveal a progressive shift in the nitrotyrosyl p*K* with increasing net positive charge of the copolymer (*Table 1*). The low pK_a of the nitrotyrosyl residue in nitrocarboxypeptidase is seemingly related to its proximity to a positive charge

on the enzyme. Thus, apparently, in solution nitrotyrosyl-248 is near a positive charge.

By analogy to arsanilazotyr-248 carboxypeptidase, this positive charge could well be the active site zinc ion. In that case, a change in physical state should affect the ionization behaviour of nitrocarboxypeptidase, as is, indeed, the case. Titration of nitrocarboxypeptidase crystals from pH 6.5 to 9.5 again increases absorbance at 428 nm while decreasing that at 360 nm. Again, the isosbestic point is at 381 nm (*Figure 6*). However, in contrast to the enzyme in solution, these data now follow a titration curve (*Figure 6*, inset) with an apparent p*K* of 8.2, rather than 6.3, a shift of almost 2 pH units. Solely the change in the physical state of the enzyme brings about this dramatic shift in titration behaviour. Thus, both in nitrocarboxypeptidase and in arsanilazocarboxypeptidase, the conformations of Tyr-248 in solution and in the crystalline state are different.

KINETIC STUDIES

Over the past 25 years, numerous studies have emphasized the remarkably complex kinetic behaviour of carboxypeptidase A in solution: it deviates from classical Michaelis–Menten kinetics when hydrolysing di- and depsipeptides, particularly carbobenzoxyglycyl-L-phenylalanine, benzoylglycyl-L-phenylalanine and benzoylglycyl-L-phenyllactate, commonly employed because of their high turnover numbers[32]. These substrates exhibit various combinations of substrate and product inhibition and/or activation reflecting the topological organization of the active centre and its modes of interaction with substrates[20, 33]. A model for substrate and product binding has placed these findings on a common basis and predicted that the use of longer substrates would eliminate such kinetic anomalies[34, 35]. This was verified by the subsequent synthesis and kinetic analysis of the hydrolysis of tri- and tetra-peptides and their ester analogues which are characterized by normal Michaelis–Menten kinetics[36–38].

In order to translate such functional data from solution kinetics into structural information, models of substrates, products and inhibitors have been built into the three-dimensional structure of the enzyme as discerned from x-ray analysis of crystals. This procedure has been thought to visualize substrate binding directly and to identify catalytic and mechanistic features of the active centre[19]. Throughout, such correlations presume that functional properties of carboxypeptidase A in solution can be interpreted in terms of its known crystal structure, based on the hypothesis that its conformations in the two states are identical.

However, as already discussed, studies employing a number of spectrochemical probes have all shown that they are different. Thus, either nitration of Tyr-248 or coupling it with diazoarsanilic acid reveals that in solution the location of this residue with respect to the zinc atom differs from that in the crystal[15, 16, 24]. Moreover, even prior to the x-ray analysis of the structure, the activity of enzyme crystals was found to be much lower than that of its solutions, strongly suggesting the preponderance of different conformations in the two physical states[7–9]. Since explicit analyses of the detailed kinetics governing crystal catalysed hydrolysis have not been reported, we have now

determined the kinetic parameters of carboxypeptidase α and γ when acting on a series of substrates[26, 27].

On going from solutions to crystals of both the α and γ enzymes for all substrates examined, activity is markedly reduced (*Table 2*), owing to a decrease of the catalytic rate constant, k_{cat}. Moreover, under otherwise

Table 2. Activities of crystals (V_c) and solutions (V_s) on hydrolysis of peptide and ester substrates

Substrate*	V_c/V_s	Substrate*	V_c/V_s
CbzGly-L-Phe	0.03	BzGlyGly-L-Phe	0.04
CbzGlyGly-L-Phe	0.05	BzGly-L-OPhe	0.01
BzGly-L-Phe	0.03	BzGlyGly-L-OPhe	0.002

* Cbz, carbobenzoxy; Bz, benzoyl; Gly, glycine; Phe, phenylalanine; OPhe, phenyllactate.

identical conditions crystallization of the enzyme can drastically alter the kinetics for any given substrate compared to those pertaining in solution. Thus, for benzoylglycylglycyl-L-phenylalanine normal kinetics in solution acquire substrate activation; for carbobenzoxyglycylglycyl-L-phenylalanine substrate inhibition inverts to activation and, remarkably, the characteristically complex substrate inhibition of benzoylglycyl-L-phenyllactate in solution is replaced by normal kinetics in the crystals. Thus, over and above the diminution of k_{cat}, crystallization of the enzyme markedly alters productive and non-productive peptide and ester binding to carboxypeptidase $A_{\alpha,\gamma}$, consistent with the spectral and chemical evidence for the existence of different conformations of the active centre in the two physical states[15, 16, 24, 25]. These kinetic data constitute, in fact, telling evidence of the functional consequences of such conformational differences thereby making available novel indices for their detection. As a consequence, x-ray structure analysis of carboxypeptidase cannot rely on kinetic data obtained solely in solution in order to be able to design valid mechanisms.

REFERENCES

[1] K. U. Linderstrøm-Lang and J. A. Schellman. *The Enzymes*, eds P. D. Boyer, H. Lardy and K. Myrbäck, Vol. I, pp 443–510. Academic Press: New York (1959).
[2] M. F. Perutz, H. Muirhead, J. M. Cox and L. C. G. Goaman. *Nature, London*, **219**, 131 (1968).
[3] L. Fretto and F. H. Strickland. *Biochim. Biophys. Acta*, **235**, 473 (1971).
[4] A. Arnone, C. J. Bier, F. A. Cotton, V. W. Day, E. E. Hazen Jr, C. D. Richardson, J. S. Richardson, and in part, A. Yonath. *J. Biol. Chem.* **246**, 2302 (1971).
[5] T. Takano, R. Swanson, O. B. Kallai and R. E. Dickerson. *Cold Spring Harbor Symp. Quant. Biol.* **36**, 397 (1971).
[6] J. A. Rupley. *Structure and Stability of Biological Macromolecules*, pp 291–352. Eds S. N. Timasheff and G. D. Fasman, Marcel Dekker: New York (1969).
[7] F. A. Quiocho and F. M. Richards. *Proc. Nat. Acad. Sci. Wash.* **52**, 833 (1964).
[8] F. A. Quiocho and F. M. Richards. *Biochemistry*, **5**, 4062 (1966).
[9] F. A. Quiocho, W. H. Bishop and F. M. Richards. *Proc. Nat. Acad. Sci. Wash.* **57**, 525 (1967).
[10] B. L. Vallee, J. F. Riordan, J. T. Johansen and D. M. Livingston. *Cold Spring Harbor Symp. Quant. Biol.* **36**, 517 (1971).
[11] J. F. Riordan, M. Sokolovsky and B. L. Vallee. *Biochemistry*, **6**, 358 (1967).
[12] G. F. Fairclough Jr and B. L. Vallee. *Biochemistry*, **9**, 4087 (1970).

[13] G. F. Fairclough Jr and B. L. Vallee. *Biochemistry*, **10**, 2470 (1971).
[14] J. T. Johansen and B. L. Vallee. *Fed. Proc.* **30**, 1108 (1971).
[15] J. T. Johansen and B. L. Vallee. *Proc. Nat. Acad. Sci. Wash.* **68**, 2532 (1971).
[16] J. T. Johansen and B. L. Vallee. *Proc. Nat. Acad. Sci. Wash.* **70**, 2006 (1973).
[17] J. T. Johansen, D. M. Livingston and B. L. Vallee. *Biochemistry*, **11**, 2584 (1972).
[18] R. G. Anderson and G. Nickless. *Analyst (London)*, **92**, 207 (1967).
[19] W. N. Lipscomb, J. A. Hartsuck, G. N. Reeke, F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead and J. C. Coppola. *Brookhaven Symp. Biol.* **21**, 24 (1968).
[20] B. L. Vallee and J. F. Riordan. *Brookhaven Symp. Biol.* **21**, 91 (1968).
[21] H. Neurath, R. A. Bradshaw, L. H. Ericsson, D. R. Babin, P. H. Petra and K. A. Walsh. *Brookhaven Symp. Biol.* **21**, 1 (1968).
[22] F. A. Quiocho and W. N. Lipscomb. *Advances in Protein Chemistry*, Vol. 25, pp 1–78. eds J. T. Edsall, C. B. Anfinsen and F. M. Richards, Academic Press: New York (1971).
[23] F. A. Quiocho, C. H. McMurray and W. N. Lipscomb. *Proc. Nat. Acad. Sci. Wash.* **69**, 2850 (1972).
[24] J. F. Riordan and G. Muszynska. *Biochem. Biophys. Res. Commun.* **57**, 447 (1974).
[25] N. Shaklai, N. Zisapel and M. Sokolovsky. *Proc. Nat. Acad. Sci. Wash.* **70**, 2025 (1973).
[26] C. Spilburg, *Fed. Proc.* **33**, 1529 (1974).
[27] C. Spilburg, J. L. Bethune and B. L. Vallee, *Proc. Nat. Acad. Sci., Wash.* **71**, 3922 (1974).
[28] M. Tabachnick and L. Sobotka. *J. Biol. Chem.* **234**, 1926 (1959).
[29] J. T. Johansen, S. Sung, I. Legg and B. L. Vallee. *Fed. Proc.* **32**, 466 (1973).
[30] W. N. Lipscomb. *Proc. Nat. Acad. Sci. Wash.* **70**, 3797 (1973).
[31] G. Muszynska and J. F. Riordan. *Fed. Proc.* **32**, 1371 (1973).
[32] R. C. Davies, J. F. Riordan, D. S. Auld and B. L. Vallee. *Biochemistry*, **7**, 1090 (1968).
[33] B. L. Vallee, J. F. Riordan, D. S. Auld and S. A. Latt. *Phil. Trans. Roy. Soc. Lond.* **B257**, 215 (1970).
[34] B. L. Vallee. *Proc. VII Intern. Congr. Biochem (Tokyo)*, 149 (1967).
[35] B. L. Vallee, J. F. Riordan, J. L. Bethune, T. L. Coombs, D. S. Auld and M. Sokolovsky. *Biochemistry*, **7**, 3547 (1968).
[36] D. S. Auld and B. L. Vallee *Biochemistry*, **9**, 602 (1970).
[37] D. S. Auld and B. L. Vallee. *Biochemistry*, **9**, 4352 (1970).
[38] D. S. Auld and B. L. Vallee. *Biochemistry*, **10**, 2892 (1971).