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Intramolecular arsanilazotyrosine-248·Zn complex of carboxypeptidase A: A monitor of catalytic events

(intramolecular coordination complex/temperature-jump stopped-flow kinetics/spectral probe/catalytic mechanism)

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ABSTRACT **The intensely chromophoric intramolecular coordination complex formed between arsanilazotyrosine-248 and the active site zinc atom of azocarboxypeptidase A (Johansen, J. T. & Vallee, B. L. (1971) *Proc. Nat. Acad. Sci. USA* 68, 2532–2535) is a spectrokinetic probe of catalytic events. The interconversion of the azoTyr-248·Zn complex and its constituents is measured by stopped-flow pH and temperature-jump methods. The rate of interconversion, 64,000 sec^{-1}, is orders of magnitude faster than that of the catalytic step itself (about 0.01–100 sec^{-1}). Rapidly turned over peptide and ester substrates disrupt the azoTyr-248·Zn complex before hydrolysis occurs. As a consequence, formation of azoTyr-248, substrate binding, and catalysis can all be monitored while catalysis is actually in progress. The results of these dynamic studies specify a course of catalytic events, different from those postulated based on x-ray structure analysis. If azoTyr-248 is displaced, the direction is opposite to the inward movement postulated on the basis of x-ray studies and is not unique to induction by substrates, since rapid changes in pH also result in analogous spectral changes.**

AzoTyr-248 carboxypeptidase has all the features which are essential for mechanistic studies: (*1*) It is enzymatically active; (*2*) the spectra of the metal complex differ characteristically from those of its constituents; (*3*) it responds dynamically to environmental factors; and (*4*) the response time of the probe itself is much more rapid than is required for the measurement of the catalytic step. These combined kinetic and spectral properties of the metal complex render it a powerful spectrokinetic probe to visualize and discern microscopic details of the catalytic process.

Spectral probes have proven very effective in exploring the relationship of catalytic activity to local structure of enzymes (1). They must be able to detect local changes in conformation, essential to and synchronous with catalysis, and must have highly specific characteristics with extraordinarily fast response times. We have employed site-specific inorganic and organic enzyme modifications to result in such sensors of electronic, magnetic, and structural changes accompanying catalysis (1). The intensely chromophoric intramolecular coordination complex between arsanilazotyrosine-248 and the zinc atom of azocarboxypeptidase (2, 3) has proven particularly informative in probing the vicinal perturbations, mutual orientation of, and distance between these two constituents of the active site of this enzyme. The spectra of this coordination complex respond dynamically to environmental factors, e.g., pH, substrates, inhibitors, and denaturing agents, as well as the physical state of the enzyme (4). Moreover, the spectra of the metal complex differ characteristically from those of its constituents and, further, the enzyme is fully active.

In the present study temperature-jump and stopped-flow techniques have served to examine the rates of interaction of azoTyr-248 with the zinc atom and of substrates with the complex. The forward and reverse rates of formation of the azoTyr-248·zinc complex are much faster than those of substrate binding and catalysis. Hence the spectra of the intramolecular coordination complex respond rapidly enough to monitor events occurring during catalysis, demonstrating a syncatalytic (1) displacement of azoTyr-248 from the zinc atom of azocarboxypeptidase by substrates. These results and interpretations are at variance with the stopped-flow studies of azocarboxypeptidase solutions previously reported (5). Further, the data are inconsistent with the substrate-induced inward movement of tyrosine-248 considered obligatory to the mechanism of action of the enzyme based on x-ray structure analysis (6–8). A preliminary account of this work has been presented (9).

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MATERIALS AND METHODS

Carboxypeptidase A_α (Sigma Chemical Corp.) and A_γ (Worthington Biochemical Corp.) were modified with diazotized arsanilic acid according to published procedures (2–4). Both enzyme forms gave analogous results. The synthesis and properties of oligopeptide substrates and their depsipeptide analogs have been described (10, 11). All other chemicals were reagent grade. Precautions to prevent contamination by adventitious metal ions (12) were taken throughout.

Stock solutions of azoenzyme, 5×10^{-4} M, were prepared in 1 M NaCl, pH 7, and after centrifugation diluted into appropriate degassed solutions prior to stopped-flow or temperature-jump experiments.

Stopped-flow experiments were performed with a Durrum-Gibson instrument equipped with a Durrum fluorescence accessory no. 16400, a 75 W xenon lamp and an end-on EMI 9526B photomultiplier. The instrument was calibrated with a Cary 14 recording spectrophotometer to yield analogous spectra under rapid kinetic conditions. Dr. Thomas C. Bruice kindly provided us access to a Durrum stopped-flow instrument equipped to measure absorbance directly, which yielded identical results.

The essential components and operation of the temperature-jump apparatus have been described (13). In the present version, its speed was increased to enable measuring a heating rate constant of 150,000 sec^{-1}. Typically, the signal-to-noise ratio was 10,000:1. Dr. Gordon Hammes kindly also placed his instrument at our disposal. The results were identical with those obtained in our laboratory.

RESULTS

We have previously detailed the pH dependence of the absorption spectral characteristics of arsanilazoTyr-248 zinc

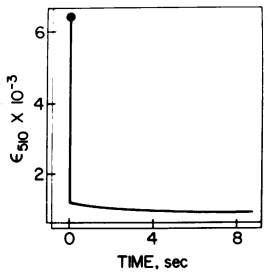

FIG. 1. Stopped-flow jump of azoTyr-248-carboxypeptidase A from pH 8.5 to 6.0. The circle (●), top left, represents the absorbance of the azoTyr-248-Zn complex at zero time, the vertical line the rapid absorbance change on mixing. The curved, horizontal line following mixing, at the bottom, is a slower process. Final conditions: enzyme, 20 μM, pH 6.0, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), 1.0 M NaCl, 25°.

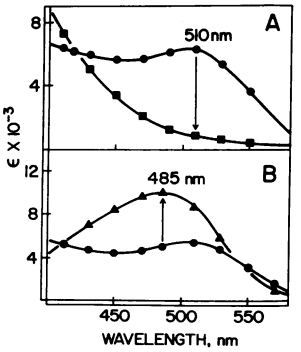

FIG. 2. Spectra of azoTyr-248-carboxypeptidase A, reconstructed from stopped-flow pH-jump experiments performed at 20 nm intervals. (A) From pH 8.5 to 6.0. Spectrum of the azoTyr-248-Zn complex at time zero (●) and that of the yellow azophenol observed within <3 msec (■). (B) From pH 8.5 to 10.5. Spectrum of the azoTyr-248-Zn complex at time zero (●) and that of the azophenolate ion observed within <3 msec (▲). Final conditions: enzyme, 20 μM, 1.0 M NaCl, 25°.

carboxypeptidase† (2–4). The spectrum of the azoTyr-248 predominates between pH 6.0 and 7.7, while that of the azophenolate ion appears between 9.5 and 10.5. Two pK_{app}'s, 7.7 and 9.5, characterize the formation and dissociation of an azoTyr-248-Zn complex which is formed maximally at pH 8.5 (3, 4). The rates of interconversion of these three species have now been determined through stopped-flow and temperature-jump experiments to establish a basis for mechanistic studies.

The response time of a spectral probe, capable of monitoring binding and/or catalytic steps, must be significantly shorter than the duration of the event to be investigated (1). Enzymatic catalysis of peptide hydrolysis generally occurs within milliseconds to seconds while binding can occur in a few microseconds (11, 14). Hence, the catalytic and binding steps of carboxypeptidase-mediated reactions can be studied by stopped-flow and temperature-jump methods, respectively. Since substrates might interact to varying degrees either with the azophenol, the complex, or the azophenolate ion, the response time of the probe to environmental factors, e.g., pH, must be known, if catalytic events are to be identified.

The conversion of the azoTyr-248-Zn complex either to the azophenol or the azophenolate species was first examined by rapidly mixing weakly buffered enzyme with concentrated buffer solutions at appropriate pH values in stopped-flow experiments. At pH 8.5, azoTyr-248 and zinc interact maximally to form the intramolecular azoTyr-248-Zn complex, but at pH 6 azoTyr-248 is almost completely formed (3, 4). Hence, when red enzyme at pH 8.5 is mixed with pH 6.0 buffer the yellow azophenol species forms instantaneously (Fig. 1). Based on measurements at 510 nm, more than 97% of the total change in absorbance requires less than 3 msec, the mixing time of the stopped-flow spectrophotometer, i.e., dissociation is extremely rapid. The remaining 3% changes more slowly, and the pertinent events have been examined in detail and will be reported (19).

Identical stopped-flow experiments have been performed over the range of wavelengths from 410 to 570 nm, thereby reconstructing the spectrum of the enzyme species present immediately after mixing with pH 6.0 buffer. This spectrum is identical with that of azotyrosine (Fig. 2A). Thus, changes in absorbance at 510 nm typify the interconversion of the red azoTyr-248-Zn complex and the yellow azotyrosine.

Similarly, mixing enzyme at pH 8.5 with pH 10.5 buffer converts the red azoTyr-248-Zn complex into the orange azophenolate ion (Fig. 2B) (3, 4). Again, dissociation is complete within the 3 msec mixing time of the instrument, except that now the resultant spectrum identifies the free azophenolate species (Fig. 2B). Hence, conversion of the azoTyr-248-Zn complex to form either the free azophenolate ion or the azophenol form is much faster than these experiments can resolve. As is apparent from previous studies at equilibrium at 510 nm‡, the conversion of the metal complex to the free azophenolate ion *increases*, but that to the azophenol form *decreases* absorbance (3, 4). Stopped-flow pH-jump experiments have been performed by varying enzyme (0.3–3.0 mg/ml) and buffer (0.01–0.1 M) concentrations, ionic strengths (0.2–1.0 M), and temperatures (5°–25°). The results are identical in all cases.

The rate of interconversion of the azoTyr-248-Zn complex to the azophenol form is resolved by means of temperature-jump, which in effect performs pH jump in a time interval much shorter than the 3 *msec* mixing time of the stopped-flow spectrophotometer. Toward this end, a solution buffered at pH 8.5 is perturbed by temperature jump, employing an instrument with a heating time of 7 μsec. On temperature jump the transmittance of the azoTyr-Zn complex at 510 nm increases rapidly, characteristic of the conversion of the red complex to the yellow azotyrosine (Fig. 3). The rate constant is 64,000 sec^{-1}, distinctly different from that for the heating process, 150,000 sec^{-1}. The magnitude of the rate constant for the interconversion of the red and yellow species is of the same order as that of many zinc complex ions (15, 16).

In previous stopped-flow experiments Lipscomb and collaborators (5) have recognized only one single exponential rate of 6.1 sec^{-1} or 7.2 sec^{-1} at enzyme concentrations of 3.0 and 1.6 mg/ml. The failure to detect the rapid process observed here led to the postulation of a number of explanations intended to account for the detection of only a single

† In order to simplify presentation, zinc arsanilazocarboxypeptidase, azocarboxypeptidase, and azoTyr-248-carboxypeptidase are used interchangeably with zinc monoarsanilazotyrosine-248 carboxypeptidase, of enzymes forms α or γ. AzoTyr-248 refers to the azophenol of monoarsanilazotyrosine-248 and the azophenolate to its ionized species.

‡ The λ_{max} for the azophenolate species is 485 nm. The molar absorptivities at 510 nm in 1 M NaCl, 25°, for the azophenol, azoTyr-Zn complex, and azophenolate species are 650, 7500, and 9500 cm^{-1} M^{-1}, respectively.

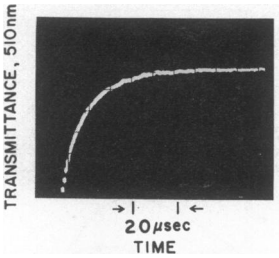

FIG. 3. Temperature jump of azoTyr-248-carboxypeptidase A and concomitant relaxation effect: enzyme, 20 μM, pH 8.5, 0.1 M Tris·HCl, 1 M NaCl. Initial temperature 21°, pulsed by 4° to result in a final temperature of 25°.

slow rate (5). The present observations of a very fast rate show that the premise for those conjectures is not valid.

Since the change in absorbance at 510 nm signals the chemical characteristics of the enzyme species present in response to environmental factors such as pH, denaturants, substrates, inhibitors, and physical state (4), it should also syncatalytically signal details of their identity at the moment of catalysis.

The rate of the interconversion of the azoTyr-248·Zn complex and azoTyr-248 is orders of magnitude faster than the catalytic rate constant for hydrolysis of either esters or peptides (10, 11). Hence, the rate of dissociation of the complex is sufficiently rapid to signal enzyme-substrate interactions. For studying these interactions, the peptide substrate Cbz-Gly-Gly-L-Phe, 0.02 M, hydrolyzed with a k_{cat} of 50 sec^{-1}, is mixed with enzyme, both at pH 8.5, where the azoTyr-248·Zn complex is maximally formed. This interaction also instantaneously decreases the absorbance at 510 nm to that characteristic of the yellow azotyrosine (Fig. 4). Under these conditions there is no evidence of a second, slow process with a rate constant of 6.1 or 7.2 sec^{-1} (5). Such a rate would clearly be too slow to account for most peptide hydrolysis, even though the contrary has been suggested (5). Obviously, it does not reflect the pH-dependent interconversion of the red and yellow species: since pH remains constant at 8.5, the rapid absorbance change observed here is entirely due to the presence of Cbz-Gly-Gly-L-Phe, not to a change in pH. Nor is it due to product formation, since immediately after mixing >99.9% of the substrate is still present, confirming that peptide hydrolysis cannot account for the observations (Table 1). Thus, this substrate displaces the azotyrosine from the zinc atom long before significant hydrolysis can occur (Table 1).

The effects of a number of peptide and ester substrates of

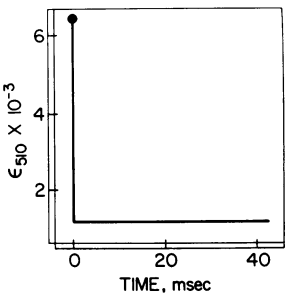

FIG. 4. Results of stopped-flow mixing azoTyr-248 carboxypeptidase with Cbz-$(Gly)_2$-L-Phe, both at pH 8.5. The circle (●), top left, represents the absorbance of the azoTyr-248·Zn complex at time zero, the vertical line, the conversion to azophenol in <3 msec. The pH remains constant at 8.5. Final conditions: enzyme, 20 μM, substrate, 50 mM, pH 8.5, 50 mM Tris·HCl, 1 M NaCl, 25°.

Table 1. Stopped-flow mixing of azoTyr-248·Zn complex with substrates at pH 8.5* resulting in azophenol formation in <3 msec

Substrate	[S], mM	$[ES]/[E]_T$	Azophenol formed, % of [ES]	Substrate remaining after 3 msec, %
Cbz-Gly-L-Phe	20	0.91	100	>99.9
Cbz-$(Gly)_2$-L-Phe	20	0.97	100	>99.9
Bz-$(Gly)_2$-L-Phe	10	0.88	98	>99.9
Bz-$(Gly)_2$-L-OPhe†	7	0.87	102	>99.9

* Final conditions: enzyme 20 μM, pH 8.5, 50 mM Tris·HCl, 1 M NaCl, 25°.

† The ester analog of Bz-$(Gly)_2$-L-Phe, with phenyllactate denoted by OPhe.

varying lengths and blocked with different groups have been studied in similar fashion with completely analogous results. In each instance the proportion of the red complex, which is converted to the yellow azophenol, is equivalent to the amount of substrate bound to the enzyme (Table 1). With all substrates, formation of the yellow azotyrosine species is complete in less than the 3 msec mixing time, while more than 99.9% of the initial peptide or ester substrate remains. The products of these enzymatic reactions, L-phenylalanine and L-phenyllactate, and their close analog, the inhibitor β-phenylpropionate, also form yellow complexes, immediately upon mixing with the azoTyr·Zn complex (Table 2). This further supports the conclusions reached on the basis of studies with substrates (Table 1).

Using equilibrium temperature-jump perturbation, the rate of substrate binding can be measured also. At pH 8.5, Gly-L-Tyr disrupts the red azoTyr-248·Zn to form the yellow azotyrosine species (4). Since the increase in temperature releases the substrate, this is promptly followed by reassociation of the residue with zinc to restore the red complex. Now, as expected, the transmittance at 510 nm decreases due to the release of substrate (Fig. 5). The rate constant for this relaxation process, 440 sec^{-1}, is more than 100 times slower than that of the response of the probe itself to temperature, 64,000 sec^{-1}. This rate constant, 440 sec^{-1}, however, is 10,000 times greater than the value of k_{cat} for this substrate, 0.01 sec^{-1}. Thus, the characteristics of this probe permit exploration of the details of the catalytic process for this and other substrates.

These data demonstrate that substrates displace azotyrosine much more rapidly from the azoTyr-248·Zn complex than substrates are hydrolyzed. As a consequence, the spectrum of this complex serves as a spectrokinetic probe of catalytic events. Hence, azotyrosine displacement, substrate

Table 2. Stopped-flow mixing of azoTyr-248·Zn complex with inhibitors at pH 8.5* resulting in azophenol formation in <3 msec

Inhibitor	[I], mM	$[EI]/[E]_T$	Azophenol formed, % of [EI]
L-Phenylalanine	50	0.95	101
L-Phenyllactate	50	0.97	99
β-Phenylpropionate	50	0.88	99

* Final conditions: enzyme, 20 μM, pH 8.5, 50 mM Tris·HCl, 1 M NaCl, 25°.

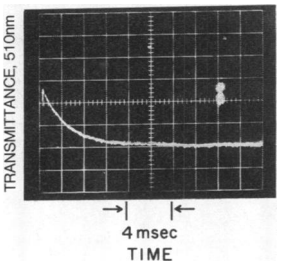

FIG. 5. Temperature jump of azoTyr-248-carboxypeptidase A in the presence of Gly-L-Tyr at pH 8.5: enzyme, 20 μM, Gly-L-Tyr, 0.8 mM, 0.1 M Tris·HCl, 1 M NaCl. Initial, final, and temperature pulse as in Fig. 3.

binding, and catalysis are all monitored syncatalytically, i.e., at the very moment of catalysis.

DISCUSSION

Only one stopped-flow kinetic study of arsanilazocarboxypeptidase has been performed previously (5). It dealt solely with the effect of pH on the interconversion of the red and yellow forms. A single, slow exponential rate, 6.1 or 7.2 sec^{-1}, dependent on conditions, was thought to reflect an ionization-induced conformational interconversion of the yellow and red forms of arsanilazotyrosine, reflecting some slow, rate-limiting, but unidentified process. It was also conjectured that its relationship to catalysis would be rate limiting, perhaps consistent with the rates of hydrolysis of peptides (5).

The present data are inconsistent with either conclusion. Essentially, we have observed rates much faster than those noted by Lipscomb and collaborators (5), obviating the experimental basis of past conjectures and, hence, the conjectures themselves.

These data also bear on deductions regarding the mechanism, based on x-ray structure analysis. The position of Tyr-248 relative to the zinc atom of the enzyme has been the subject of much speculation in the past (5–8, 17). The data presented here and elsewhere (2–4) leave little doubt, that, in solution, both substrate binding and change in pH disrupt the azoTyr-248·Zn complex. X-ray analysis of the native enzyme at pH 7.5 has placed Tyr-248 17 Å away from the zinc atom and, further, any interaction between them was thought unlikely (5). Gly-L-Tyr was postulated to induce an obligatory movement of Tyr-248 inward toward the zinc atom, and both the process and its direction were considered critical to the mechanism of action (5–8). These deductions from structure studies of crystals, already discussed, were thought to be reinforced by the results of the previous pH-jump experiments (5).

The stopped-flow pH and temperature-jump studies of the azoTyr-248·Zn complex, together with spectral studies at equilibrium (2–4) and the kinetic properties of carboxypeptidase crystals (18), lead us to quite different conclusions. Both substrate and pH alter the position of the azoTyr-248 relative to the zinc atom; if there is any major movement, its direction must be away from the zinc atom (Figs. 1–5). Moreover, such a movement would then not be unique to the effect of changes brought about by substrates, since changes of pH result in analogous spectral effects. Hence, the results of these dynamic as well as other studies (4) specify a course of catalytic events different from those postulated, based on x-ray structure analysis (5–8, 17).

It is apparent that an inward movement of tyrosine-248 toward the zinc atom cannot be an obligatory feature of the mechanism of action of the enzyme. Further, the data lend no support to the view that the interaction of Tyr-248 and zinc would result in an inactive species (17): arsanilazoTyr-248 zinc carboxypeptidase is fully active in solution (4).

We have previously described the remarkable capacity of the arsanilazocarboxypeptidase chromophore to help visualize the differences of conformations of this enzyme in crystals and solutions. Thus, the unique circular dichroism negative extremum at 510 nm reflects the mutual topography of azoTyr-248 and zinc, the two constituents of the active center considered indispensable to function (5). The present rapid flow and temperature-jump kinetic studies demonstrate that the kinetic and spectral properties of the intramolecular coordination complex combine to render it an equally powerful probe to visualize and discern microscopic details of the catalytic process.

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1. Vallee, B. L., Riordan, J. F., Johansen, J. T. & Livingston, D. M. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 517–531.
2. Johansen, J. T. & Vallee, B. L. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2532–2535.
3. Johansen, J. T., & Vallee, B. L. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2006–2010.
4. Johansen, J. T. & Vallee, B. L. (1975) *Biochemistry* **14**, 649–660, and references therein.
5. Quiocho, F. A., McMurray, C. H. & Lipscomb, W. H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2850–2854.
6. Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. & Coppola, J. C. (1968) *Brookhaven Symp. Biol.* **21**, 24–90.
7. Lipscomb, W. N., Reeke, G. N., Hartsuck, T. A., Quiocho, F. A. & Bethge, P. H. (1970) *Phil. Trans. R. Soc. London Ser. B* **257**, 177–214.
8. Quiocho, F. A. & Lipscomb, W. N. (1971) in *Advances in Protein Chemistry*, eds. Edsall, J. T., Anfinsen, C. B. & Richards, F. M. (Academic Press, Inc., New York), Vol. 25, pp. 1–78.
9. Harrison, L. W., Auld, D. S. & Vallee, B. L. (1975) *Fed. Proc.* **34**, 522, abstr. 1698.
10. Auld, D. S. & Vallee, B. L. (1970) *Biochemistry* **9**, 602–609.
11. Auld, D. S. & Holmquist, B. (1974) *Biochemistry* **13**, 4355–4361.
12. Thiers, R. E. (1957) in *Methods of Biochemical Analysis*, ed. Glick, E. (Interscience Publishers, Inc., New York), Vol. 5, pp. 273–335.
13. French, T. C., Yu, N-T. & Auld, D. S. (1974) *Biochemistry* **13**, 2877–2882.
14. Hess, G. P. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, Inc., New York), Vol. 3, 3rd ed., pp. 213–248.
15. Miceli, J. A. & Stuehr, J. E. (1972) *Inorg. Chem.* **11**, 2763–2767.
16. Wilkins, R. G. (1964) *Inorg. Chem.* **3**, 520–522.
17. Lipscomb, W. N. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3797–3801.
18. Spilburg, C. A., Bethune, J. L. & Vallee, B. L. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3922–3926.
19. Harrison, L. W., Auld, D. S. & Vallee, B. L. (1975) *Proc. Nat. Acad. Sci. USA* **72**, in press.