ARSANILAZOCARBOXYPEPTIDASE A: ENVIRONMENTALLY SENSITIVE EXTRINSIC COTTON EFFECTS*

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Coupling of carboxypeptidase A with p-azobenzenearsonate generates an absorption spectrum which gives rise to at least three extrinsic Cotton effects between 300 and 600 mμ. These, like the activity changes accompanying the chemical modifications, are consistent with covalently formed arsanilazotyrosine in the enzyme. Further, the Cotton effects are altered significantly by the binding of substrates or inhibitors, rendering the arsanilazotyrosyl chromophore an optically active probe of the active center.

Introduction

The functional role of tyrosyl residues in carboxypeptidase A catalysis has been demonstrated through a number of chemical modifications (1). Acetylation, iodination and azocoupling of tyrosine all alter the esterase and peptidase activities of the enzyme, as does nitration, which in addition, generates a visible absorption spectrum sensitive to the environment of the active center (2). Modification of carboxypeptidase A with p-azobenzenearsonate also changes the activity of this enzyme and generates an absorption spectrum which-in this case-is optically active and moreover, altered by the binding of substrate or inhibitor.

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Experimental

Carboxypeptidase A_γ was obtained from the Worthington Biochemical corporation. Peptidase activity (3) and esterase activity (4) were assayed as described previously. Solutions of p-azobenzenearsonate were prepared by diazotization of 0.5 mmoles p-arsanilic acid (Eastman Organic Chemicals) in 10 ml of 0.3 M HCl with 0.75 mmoles $NaNO_2$ at 0°. After 30 minutes the reaction mixture was adjusted to pH 5 and diluted to 25 ml with ice-cold water. Azocoupling of carboxypeptidase (2×10^{-4} M) was carried out at 0° in 0.33 M $NaHCO_3$, 1 M NaCl, pH 8.8, by the addition of suitable aliquots of a freshly prepared solution of p-azobenzenearsonate. The coupling reaction was stopped after 40 minutes by quenching the unreacted diazo reagent with phenol, followed by dialysis versus several changes of 0.04 M Tris, 1 M NaCl, pH 7.7, at 4°. Circular dichroism (CD) spectra were measured with a Cary Model 60 spectropolarimeter over the wavelength range from 300 to 600 mμ at 25° employing cells of 0.3 to 1.0 cm light path and protein concentrations of 8×10^{-5} M and less. There was no evidence for concentration dependence or artifacts due to absorption under these conditions. Molar ellipticities [θ] are not corrected for the refractive index of the solvent (5).

Results and Discussion

Coupling of carboxypeptidase A_γ with up to a 10-fold molar excess of p-azobenzenearsonate decreases the peptidase activity to 50% of the control, but the esterase activity remains relatively unchanged (Figure 1). There is little further change when a 20-fold molar excess is employed. These alterations in catalytic activities are prevented if the inhibitor, β-phenylpropionate, is present during the modification.

p-Azobenzenearsonate is known to modify tyrosyl, lysyl and histidyl residues of proteins (6,7). Since acid hydrolysis destroys these arsanilazo derivatives without regeneration of the free amino acids, the chemical consequences of the modification can be assessed by comparing the amino acid analyses of acid hydrolysates of the native with those of the modified protein. Coupling of the enzyme with a 10-fold molar excess of reagent modifies 1.2 tyrosyl and

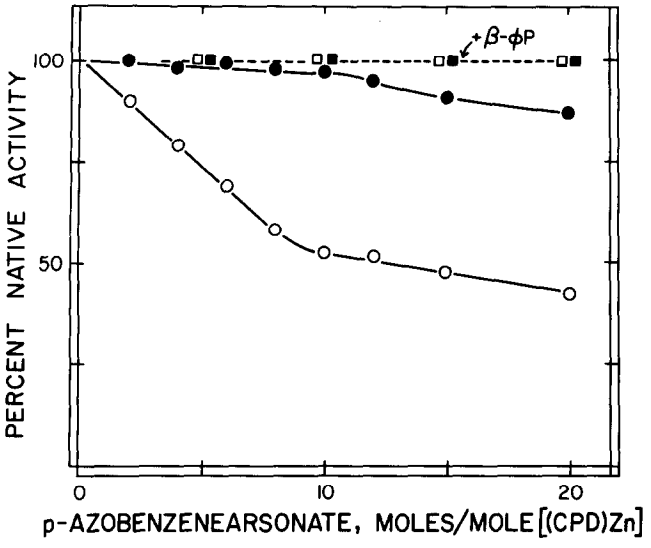

Figure 1 - Effect of coupling carboxypeptidase A with p-azobenzenearsonate on the enzymatic activities. ●——●, esterase activity; O——O, peptidase activity; — — — —, esterase or peptidase activities on coupling in the presence of 0.1 M β-phenylpropionate.

1.1 lysyl residues. All other residues including histidine are unchanged. The sum of the residues modified agrees closely with the gram atoms of arsenic incorporated into the protein, as measured by atomic absorption spectrometry, indicating further that only lysine and tyrosine are modified under these conditions (Table I).

The absorption spectrum of the enzyme modified with a 10-fold molar excess of reagent is similar to that of the model compound, monoarsanilazo-N-chloroacetyltyrosine, consistent with the formation of monoarsanilazotyrosine in the enzyme (Figure 2). Based on the absorptivities at 460 and 500 mμ at pH 13 (7), the modified protein contains 1.04 azotyrosyl and negligible (less than 0.1) azohistidyl residues per mole, consistent with the results of amino acid analyses (Table I).

In contrast to the featureless CD spectrum of the native enzyme between 300 and 600 mμ, arsanilazocarboxypeptidase exhibits multiple overlapping extrinsic Cotton effects (Figure 3). This complex spectrum contains at least

TABLE I

CHEMICAL CONSEQUENCES OF THE MODIFICATION OF CARBOXYPEPTIDASE A WITH A 10-FOLD MOLAR EXCESS OF p-AZOBENZENEARSONATE

RESIDUE	NUMBER MODIFIED[a]
Tyrosine	1.2
Lysine	1.1
Sum (Lysine plus Tyrosine)	2.3
$\frac{\text{Gram atoms As}}{\text{mole enzyme}}$	2.6[b]

[a]Determined by amino acid analysis of acid hydrolysates and expressed as the differences in residues between the native and modified protein.

[b]Determined by atomic absorption spectrometry of the dialyzed, modified enzyme.

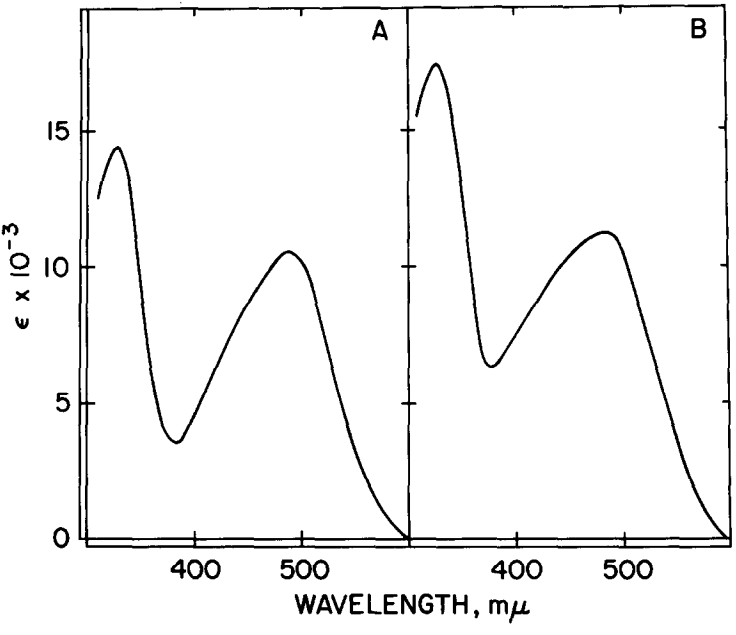

Figure 2 - Absorption spectra in 0.1 N NaOH. A. Monoarsanilazo-N-chloroacetyltyrosine; B. Arsanilazocarboxypeptidase prepared with a 10-fold molar excess of reagent.

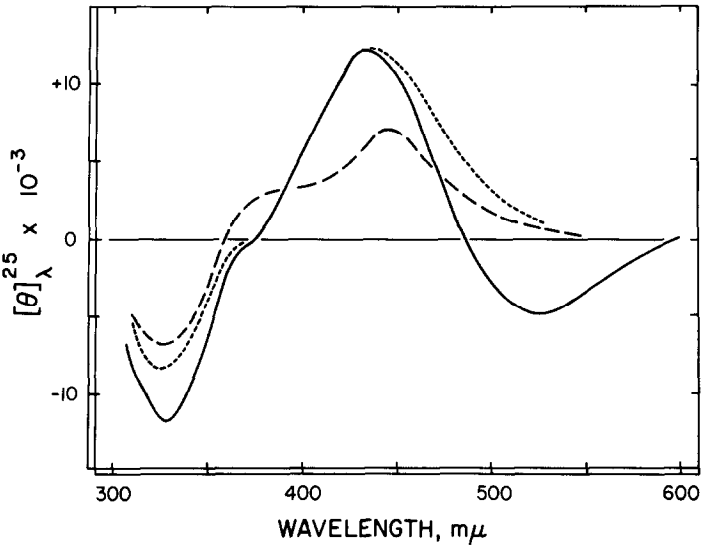

Figure 3 - Circular dichroic spectra in 0.04 M Tris, 1 M NaCl, pH 7.7. Baseline = native carboxypeptidase or arsanilazo-O-acetyl-carboxypeptidase; ———— , arsanilazocarboxypeptidase or arsanilazo-N-succinylcarboxypeptidase; — — — , arsanilazo-carboxypeptidase plus 4.33 x 10^{-3} M β-phenylpropionate; - - - - , arsanilazocarboxypeptidase plus 2.5 x 10^{-3} M glycyl-L-tyrosine.

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one positive and two negative ellipticity bands at 435, 525 and 328 mμ, respectively, which may be susceptible to further resolution. Prior O-acetylation of the free tyrosyl residues of the native enzyme largely prevents the generation of arsanilazotyrosine, as judged by spectral and CD analysis (Figure 3) However, prior protection of only the free lysyl residues by succinylation does not prevent the generation of either the absorption spectrum characteristic of arsanilazotyrosine or the extrinsic Cotton effects. Similarly, prior O-acetylation prevents the activity changes induced by modification with p-azobenzenearsonate but prior N-succinylation does not. These results suggest that both the generation of the Cotton effects as well as the alteration of enzymatic activity reflect the formation of arsanilazotyrosine in the enzyme.

Since p-azobenzenearsonate seems to modify residues which may be involved in catalytic function, it was of interest to assess the effect of agents, known to bind to the active center, on the optical activity of the modified enzyme.

Upon the addition of the inhibitor, β-phenylpropionate, the CD spectrum of arsanilazocarboxypeptidase undergoes marked changes (Figure 3). The negative band at 525 mμ disappears completely, the amplitudes of the positive and negative dichroic bands at 430 and 328 mμ, respectively, decrease while a second positive Cotton effect centered at 380 mμ is resolved. The addition of the peptide substrate, glycyl-L-tyrosine, to the modified enzyme causes similar though not identical changes in the CD spectrum (Figure 3). These changes in optical activity brought about by the binding of substrate or inhibitor are accompanied by lesser changes in the absorption spectrum of the azoenzyme at pH 7.7 which will be considered elsewhere (8).

Perturbation of the CD spectrum by agents known to bind at the active center suggests that such binding may induce direct or indirect alterations in the conformation of the arsanilazotyrosyl chromophore generated by the modification. The present results are consistent with earlier studies of nitrocarboxypeptidase (2) and those of x-ray diffraction of crystals which reveal the induction of a marked conformational change in a tyrosyl residue by binding of substrate or inhibitor to carboxypeptidase A_α (9).

Numerous instances of extrinsic Cotton effects have now been observed owing to prosthetic groups and reversible interactions of enzymes with substrates, substrate analogs, coenzymes, metals and inhibitors (10). However, there are as yet relatively few instances in which chemical modifications of amino acid residues have been found to induce optical asymmetry. As demonstrated here, the environmental sensitivity of optically active arsanilazotyrosine generated in carboxypeptidase A provides a useful new dimension for the study of interactions of substrates and inhibitors with this enzyme. We are currently exploring the physico-chemical bases of the observed effects and the relationship of tyrosine modified by p-azobenzenearsonate with modification of this enzyme by other reagents.

References

1. Vallee, B. L. and Riordan, J. F., Brookhaven Symposia in Biology, 21, 1969, in press.
2. Riordan, J. F., Sokolovsky, M. and Vallee, B. L., Biochemistry, 6, 358 (1967).
3. Coleman, J. E. and Vallee, B. L., J. Biol. Chem., 223, 457 (1960).
4. Simpson, R. T., Riordan, J. F. and Vallee, B. L., Biochemistry, 2, 616 (1963).
5. Djerassi, C. (ed.), Optical Rotatory Dispersion, McGraw-Hill, New York (1960).
6. Tabachnick, M. and Sobotka, H., J. Biol. Chem. 234, 1726 (1959).
7. Tabachnick, M. and Sobotka, H., J. Biol. Chem. 235, 1051 (1960).
8. Kagan, H. M. and Vallee, B. L., manuscript in preparation.
9. Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. and Cuppola, J. C., Brookhaven Symposia in Biology, 21,1969, in press.
10. Ulmer, D. D. and Vallee, B. L., Adv. in Enzymol., Vol. 27, Interscience, New York, p. 37 (1965).