ARSANILAZOPROCARBOXYPEPTIDASE ACTIVATION*

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Modification of procarboxypeptidase A with diazotized arsanilic acid under mild conditions generates extrinsic Cotton effects attributed to the 1.7 tyrosyl residues substituted. The C.D. spectrum exhibits extrema at 305, 400 and 500 nm. Activation shifts the band at 400 to 438 nm, almost doubling its molecular rotation. The 500 nm band shifts to 525 nm. The time course of the spectral changes correlate directly with that of the induction of activity. The arsanilazochromophore is thus a suitable probe of conformational changes accompanying the activation process of this trimeric zymogen.

We have reported recently that chemical modifications of proteins with diazonium salts generate extrinsic Cotton effects in the visible region of the spectrum, distinctive for each of the proteins examined (1). The resultant azochromophores appear to reflect both overall and local conformation and hence can be employed as probes for structural changes accompanying biological phenomena.

Owing to complexities, intrinsic to the molecule (2), the proteolytic events underlying the process of activation of procarboxypeptidase A have not been defined as decisively as those for certain other pancreatic zymogens, e. g., chymotrypsinogen. Procarboxypeptidase is activated by a series of enzymatically catalyzed reactions which reduce its molecular weight from 87,000 to 34,600, i. e., that of carboxypeptidase, the active enzyme (2,3). The present study demonstrates that arsanilazochromophores can be used to monitor the process of its activation.

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EXPERIMENTAL: Procarboxypeptidase was isolated from acetone-dried steer beef pancreas glands (2,3). Peptidase and esterase activities were performed as previously described (4,5). Diazotized arsanilic acid (p-azobenzenearsonate) was 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 distilled water. Coupling with procarboxypeptidase, 2×10^{-4} M, was carried out by addition of suitable aliquots of freshly prepared solution of diazotized arsanilic acid at 0° in 0.67 M $NaHCO_3$, 1 M NaCl, pH 7.7. The coupling reaction was stopped after 40 minutes by quenching the unreacted diazo reagent with phenol, 2-fold excess over the reagent, followed by dialysis *vs* four to five changes of 0.04 M Tris, 1 M NaCl, pH 7.7 at 4°. The numbers of histidyl and tyrosyl residues modified were determined by measurement of the absorption spectra in 0.1 M NaOH (6,7). Arsanilazoprocarboxypeptidase was activated using a 1/5 wt. ratio of trypsin/zymogen for 24 hours at 25 ± 0.1° in 0.04 M Tris-HCl, 0.1 M NaCl, pH 7.7, followed by extensive dialysis against the same buffer. Total incorporation of diazotized arsanilic acid into protein was determined by measurement of its arsenic content, using atomic absorption spectroscopy (8).

Arsanilazo*apo*procarboxypeptidase was prepared by incubation with a 20-fold molar excess of 1,10-phenanthroline. The zinc phenanthrolinate was then removed by gel filtration using Sephadex G-25 (9). Circular dichroic (CD) spectra were measured with a Cary Model 60 spectropolarimeter over the wavelength range from 300 to 600 nm at 25° employing cells of 0.3 to 1.0 cm light path and protein concentrations varying between 10^{-4} M and 10^{-5} M. Under these conditions, there was no evidence for concentration dependence or artifacts due to absorption. Molecular ellipticities, $[\theta]_{\lambda}^{25}$, are not corrected for the refractive index of the solvent (10).

RESULTS AND DISCUSSION: Diazotized arsanilic acid is known to modify tyrosyl, lysyl and histidyl residues of proteins (6,7). Since the arsanilazo derivatives of tyrosine, histidine and lysine are not converted to the parent amino

acids by acid hydrolysis, amino acid analysis of protein hydrolysates can be employed in conjunction with spectra and arsenic analyses to assess the chemical consequences (11) of procarboxypeptidase modification. As observed in other instances (11), under the conditions employed, only tyrosyl and lysyl residues of this zymogen are modified to a significant extent, while all other residues appear to be unaltered. The sum of the tyrosyl and lysyl residues modified agrees closely with the number of gram atoms of arsenic incorporated, further indicating that no other residues are modified (Table I).

TABLE I

CHEMICAL CONSEQUENCES OF THE
MODIFICATION OF PROCARBOXYPEPTIDASE
WITH DIAZOTIZED ARSANILIC ACID

ARSANILAZO-DERIVATIVE	SPECTRAL ANALYSIS	AMINO ACID ANALYSIS
TYROSINE	1.6 ± 0.1	1.7 ± 0.3
LYSINE	—	6.1 ± 0.3
HISTIDINE	0.51 ± 0.05	0.4 ± 0.2
Σ OF MODIFIED RESIDUES = 8.2		
$\frac{\text{GRAM ATOM As}}{\text{MOLE}}$ = 8.4		

The visible absorption spectrum of arsanilazoprocarboxypeptidase is characteristic of monoarsanilazotyrosine with a shoulder at 330 nm and a broad envelope extending to 600 nm (Figure 1A). The CD spectrum, however, consists of multiple, overlapping extrinsic Cotton effects (Figure 1B). There are at least one positive and two negative ellipticity extrema at 400, 500, and 305 nm, respectively. Such data are consistent with azocoupling of phenolic side chains in model systems (12).

Activation of the arsanilazozymogen markedly changes its CD but not its absorption spectrum (Figure 2). The positive extremum at 400 nm progressively

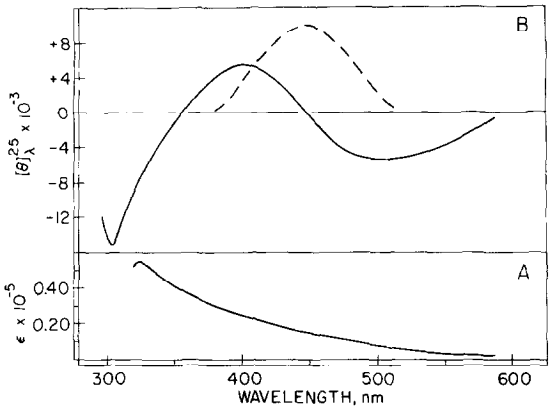

Figure 1: A) Absorption spectrum of arsanilazoprocarboxypeptidase in 0.04 M Tris-HCl, 1.0 M NaCl, pH 7.7; B) CD spectrum of arsanilazoprocarboxypeptidase (——); arsanilazoapoprocarboxypeptidase (----).

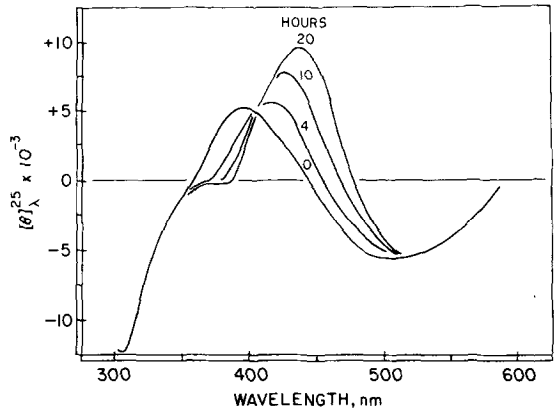

Figure 2: Measurements of circular dichroism during activation of arsanilazoprocarboxypeptidase in 0.14 M Tris-HCl, 1.0 M NaCl, pH 7.7, at times indicated.

shifts to higher wavelengths while also increasing in magnitude, to be centered at 438 nm $[\theta]_{438}^{25}$ = 10,000° after 20 hours, when activation is complete. From that time on there are virtually no further changes either in the CD spectrum or in activity; simultaneously, the negative extremum at 500 shifts to 525 nm. The period required for the CD changes, i. e., 20 hours, a typical time span for the activation of this enzyme precursor, is much longer than that of most other pancreatic zymogens (13). The spectral characteristics of the end product are closely similar to those of the arsanilazoenzyme when modified directly (11)

In conjunction with the temporal events, this suggests that the changes observed reflect the transition of the molecule from the enzymatically inert to the active state. In this regard, the appearance of esterase and peptidase activities during activation correlates closely with the CD changes (Figure 3). Thus, these probe properties appear to reflect the kinetics of alterations in structure of the molecule related to the induction of activity.

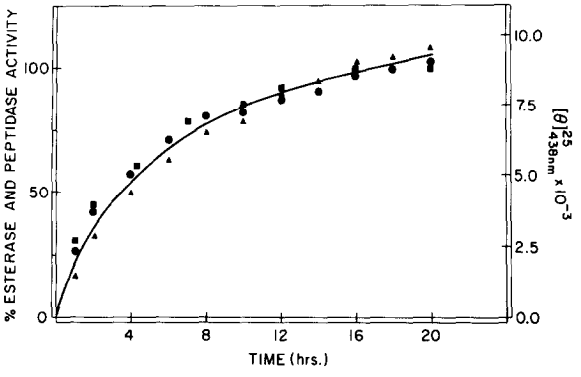

Figure 3: Correlation of the molecular ellipticity at 438 nm, $[\theta]^{25}_{438}$, (▲ — ▲), with the appearance of esterase (● — ●), and peptidase (■ — ■) activities.

The inhibitor, β-phenylpropionate, alters the CD spectrum of azocarboxypeptidase, implying conformational changes (11). The agent, 0.02 M, completely abolishes the negative extremum at 525 nm of the product of activation and diminishes the molecular ellipticity of the positive extremum at 438 nm, but it does not alter the CD spectrum of arsanilazoprocarboxypeptidase, suggesting a different mode of binding.

Removal of the catalytically essential zinc atom of the product abolishes the negative band at 525 nm and that of arsanilazozymogen at 500 nm (Figure 1B), indicating a close relationship between the arsanilazochromophore and the zinc atom, destined to become part of the active center of the enzyme.

The present data thus suggest that arsanilazochromophores can serve as probes of structural changes reflecting conformational changes in zymogen

activation. The chemical details of the activation process of most of the pancreatic zymogens have been relatively accessible both to sequence studies and X-ray structure analysis. The relatively more complex procarboxypeptidase molecule which exhibits quaternary structure has proven more resistant to the delineation of structure-function relationships by established procedures. The present approach, applicable to zymogens with subunit structure, might further provide opportunities to monitor conformational changes in enzymes with quaternary structure.

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