Some Effects of Calcium Ions on the Structure of Bovine Pancreatic Deoxyribonuclease A*

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SUMMARY

The effects of divalent metal ions on the circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of bovine pancreatic deoxyribonuclease have been studied. Both the CD and ORD spectra of DNase undergo significant changes below 250 nm upon the binding of calcium to the enzyme. The maximum increase in ellipticity at the 215 nm minimum in the CD spectrum is 950 deg cm² per decimole. Fifty percent of the maximum transition occurs near 10⁻⁵ M CaCl₂. MnCl₂ gives a smaller increase at 215 nm of 290 deg cm² per decimole while MgCl₂ causes no change.

Calcium is able to protect DNase completely against inactivation by trypsin. Half-maximum protection is achieved near 1.3 × 10⁻⁴ M CaCl₂. Although some protection against trypsin inactivation is given by MnCl₂ and less by MgCl₂, at no concentration is either metal able to protect completely. Calcium-induced changes in the CD spectrum and in susceptibility to trypsin must not involve any large changes in shape or volume of the protein since no change in the sedimentation coefficient could be observed.

Concentrations of 10⁻⁴ M to 10⁻² M CaCl₂ cause intensification of the CD bands between 310 nm and 250 nm. The same calcium concentration range induces a large ultraviolet difference spectrum in DNase. This indicates that calcium causes significant perturbations of tryptophan and tyrosine residues in DNase.

It has been established that bovine pancreatic deoxyribonuclease requires divalent metal ions to hydrolyze the phosphodiester bonds of DNA (1-4). The binding of metal ions to nucleic acids has been studied (5-7) and the DNA-metal ion complex is probably the required substrate for DNase action. Calcium ions protect one of the two disulfide bonds from reduction by mercaptoethanol (8), and Ca++ protects DNase from inactivation by trypsin and chymotrypsin (9). Calcium also alters the extent of reaction and degree of inactivation when the ε-amino groups of the 9 lysine residues are modified with trinitrobenzene sulfonic acid or potassium cyanate (10). Calcium causes a red shift in the ultraviolet adsorption spectrum of DNase indicating a calcium-induced perturbation of tryptophan and tyrosine residues (11). Divalent metal ions are necessary for alkylation of the active site histidine with iodoacetate (12).

Divalent metal ions also affect the mechanism by which DNase cleaves DNA. Douvas and Price have demonstrated that when calcium is bound to DNase, the enzyme can cleave both strands of DNA in one encounter. When magnesium alone is present, DNase cleaves exclusively by breaking only one strand of DNA per encounter. These several effects of calcium ions suggest that the binding of calcium to DNase induces some structural changes in the enzyme. We therefore wished to investigate the effects of divalent metal ions on the structure of DNase with the use of circular dichroism as our main investigative tool.

EXPERIMENTAL PROCEDURE

Materials—Worthington DP grade DNase was treated with diisopropyl fluorophosphate to inactivate contaminant proteolytic enzymes and purified over a column of phosphocellulose according to the method of Salnikow, Moore, and Stein (13). Four chromatographically distinct components of DNase emerge, and the last and largest peak, designated DNase A, was used in these studies. DNase A solutions were concentrated routinely by ultrafiltration. At no time was the protein concentrated by lyophilization. Worthington trypsin treated with L-l-tosyl-amido-2-phenylethyl chloromethyl ketone to inactivate any contaminating chymotrypsin was used without further purification.

DNase Assay—DNase activity was determined by the hyperchromicity assay of Kunitz (14) as modified by Price et al. (9) against a Mn++-DNA substrate at pH 5.0.

Optical Rotatory Dispersion and Circular Dichroism—Circular dichroism spectra were measured at room temperature in the J-10 modification of the Durrum Jasco ORD/UV 5 spectropolarimeter. Optical rotatory dispersion spectra were measured in a Durrum Jasco ORD/UV 5 instrument at room temperature. A 0.10-cm cell was used for CD and ORD measurements below 250 nm, and a 1.0-cm cell was used for CD measurements above 250 nm. The reproducibility of both spectra and baseline were checked repeatedly.

In preparation for ORD and CD runs, DNase was either desalted at room temperature over a Sephadex G-25 column equilibrated with the desired buffer or dialyzed against the desired

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buffer at 5°. The buffers used were 1 mM HCl for pH 3.0, 5 mM sodium phosphate for pH 5.5 and pH 6.5, and 5 mM ionic strength Tris-HCl for pH 7.5 and pH 8.5. To compare the ORD or CD of DNase with and without metal ions, a sample of DNase was divided into two equal volumes. To one was added a small volume (5 to 20 µl) of the divalent metal ion solution. Maximum volume changes ranged from 0.2% to 0.5%, thus making errors in calculating ellipticity and specific rotation changes due to protein concentration differences negligible.

**Ultraviolet Difference Spectra**—Difference spectra were run on a Cary model 14 spectrophotometer. Two ml of 1.45 mg per ml DNase in 5 mM ionic strength Tris-HCl buffer, pH 7.5, was delivered into each of two, 1.0-cm path length cuvettes. After establishing a protein versus protein baseline, a small volume of CaCl2 solution was added to one cuvette and an equal volume of water to the reference cuvette. After allowing several minutes for equilibration, the difference spectrum was recorded.

**Ultracentrifugation**—Ultracentrifuge runs were done at 20° in 0.1 M Tris-HCl buffer, pH 8.5, in a Spinco model E ultracentrifuge. The protein concentration was 4 to 6 mg per ml. Runs were made at 60,000 rpm. Two, 12-mm aluminum centerpiece cells were used with a 1° quartz wedge in one cell enabling the measurement of two schlieren patterns on the same photographic plate. Each cell contained DNase solutions of identical concentration and pH except one cell contained CaCl2 and the other cell a sufficient amount of NaCl to keep the ionic strength constant. Differences in the sedimentation coefficient due to calcium binding could be detected in a single ultracentrifuge run with a maximum error of 1 to 2%. This error occurs in the measurement of the photographic plates.

**RESULTS**

**Optical Rotatory Dispersion and Circular Dichroism Below 250 nm**—The ORD spectrum of DNase with and without calcium is shown in Fig. 1. The minimum at 229 nm in the absence of calcium agrees with the previously published results of Cheng (15) who observed a negative minimum at 228 ± 1 nm. When calcium is present the negative shoulder at 238 nm increases and becomes a definite negative minimum while the specific rotation at 229 nm decreases. Timasheff and Bernardi also have observed a double negative minimum in the ORD spectrum of DNase (16), suggesting that their samples of DNase may have contained calcium. Price has observed identical calcium-induced changes in the ORD spectrum of DNase with a Cary model 60 spectropolarimeter. 3

The calcium-induced ORD change occurs in the spectral region where the Cotton effects attributed to the asymmetry of the peptide backbone reach their maximum negative value due to the presence of such secondary structure as the β pleated sheet and the α helix (17). Since an ORD spectrum change occurs in this region when calcium binds to DNase, one expects a corresponding change in the CD spectrum at wavelengths characteristic of peptide bond absorption. This is substantiated by the CD spectrum of DNase with and without calcium, shown in Fig. 2. The increase in the ellipticity of the peptide bond transition at the minimum indicates a significant perturbation of the asymmetry of the peptide backbone and suggests that calcium binding results in a more ordered structure of the polypeptide backbone of DNase. The CD spectrum of DNase is also characteristic

3 P. A. Price, unpublished results.
**TABLE I**

Effects of Ca++ on Structure of DNase

The buffers used were described under "Experimental Procedure." DNase concentrations ranged from 0.12 to 0.16 mg per ml.

<table>
<thead>
<tr>
<th>CaCl₂ concentration</th>
<th>pH</th>
<th>Change in ellipticity at 215 nm in deg cm² per decimole</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻³ M</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>5 × 10⁻³ M</td>
<td>5.5</td>
<td>+725</td>
</tr>
<tr>
<td>2 × 10⁻³ M</td>
<td>6.0</td>
<td>+743</td>
</tr>
<tr>
<td>1.29 × 10⁻³ M</td>
<td>7.5</td>
<td>+950⁹</td>
</tr>
<tr>
<td>1.25 × 10⁻³ M</td>
<td>8.0</td>
<td>+950</td>
</tr>
</tbody>
</table>

*The maximum increase of 950 deg cm² per decimole is the average of several different experiments.*

**TABLE II**

Effect of MgCl₂ and MnCl₂ on ellipticity at 215 nm

The buffer used was 5 mM ionic strength Tris-HCl, pH 7.5. The DNase concentration was 0.16 mg per ml.

<table>
<thead>
<tr>
<th>Metal ion type</th>
<th>Δθ at 215 nm in deg cm² per decimole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 or 5.0 × 10⁻² M MgCl₂</td>
<td>0</td>
</tr>
<tr>
<td>1.0 or 5.0 × 10⁻² M MnCl₂</td>
<td>+290</td>
</tr>
<tr>
<td>5 × 10⁻² M MgCl₂ + 10⁻⁴ M CaCl₂</td>
<td>+950</td>
</tr>
<tr>
<td>5 × 10⁻² M MnCl₂ + 10⁻⁴ M CaCl₂</td>
<td>+950</td>
</tr>
<tr>
<td>10⁻⁴ M CaCl₂</td>
<td>+950</td>
</tr>
</tbody>
</table>

**TABLE III**

Change in ellipticity at 215 nm as function of CaCl₂ concentration

Tris-HCl buffer, 5 mM ionic strength, pH 8.5, was used. The DNase concentration was 0.13 mg per ml.

<table>
<thead>
<tr>
<th>CaCl₂ concentration</th>
<th>Δθ at 215 nm in deg cm² per decimole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>+450</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>+950</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>+950</td>
</tr>
</tbody>
</table>

at 215 nm is given in Table III. Half-maximum change occurs near 10⁻⁴ M CaCl₂. Price observed that half-maximum protection against trypsin inactivation occurs near 1.3 × 10⁻⁴ M CaCl₂ (18). Fig. 3 shows the effect of MnCl₂, MgCl₂, and CaCl₂ on the rate of inactivation due to trypsin. If one plots the logarithm of the percentage of remaining activity versus time a linear plot is obtained and relative pseudo first order rate constants can be calculated. The relative rates of inactivation calculated from the slopes of such plots are 10 for 0 ions, 1.5 for magnesium, and 0.26 for manganese. Even though manganese and magnesium slow the rate of inactivation, only calcium protects completely. Thus, calcium is the most effective of the three divalent metal ions both in protecting against trypsin and inducing the CD change. Manganese is second in both effects, and magnesium gives no CD change and is a poor protector against trypsin.

**Sedimentation Velocity Runs**—If the conformational alterations observed by the increase in the 215-nm minimum of the CD spectrum involved large alterations in shape or volume of the protein, one might expect a change in the sedimentation coefficient. The technique we used involved paired sedimentation velocity runs in the analytical ultracentrifuge with two cells in the same rotor, enabling the measurement of two schlieren patterns on the same photographic plate. Both cells contain an identical concentration of protein at the same pH and ionic strength. One cell contains CaCl₂ and the other sufficient NaCl to maintain constancy of ionic strength in both cells. The results from some typical runs are shown in Fig. 4. The slopes of the plots in Fig. 4a are identical indicating that calcium causes no change relative to the enzyme with sodium. The sedimentation coefficients we obtained from one experiment to another were in good agreement with the value of 2.78 obtained by Lindberg (19). As a control to test the accuracy of our measurements in a single experiment, paired sedimentation runs with both samples containing calcium were made (Fig. 4b). This control enabled us to estimate our maximum error to be about 1 to 2% in the measurement of the photographic plates. A different treatment of the data can be made if one plots the difference in the logarithm of the distance from the center of rotation versus time. The slopes of such a plot are a direct measure of the change in sedimentation coefficient (20), and such plots also indicate that no change in the sedimentation coefficient has occurred.
occurred. The calcium-induced CD change, therefore, must not represent any large alterations in size and shape of the molecule.

**Circular Dichroism from 310 to 250 nm**—The near ultraviolet CD of DNase has been previously published (19). Fig. 5 shows the effect of calcium on the near ultraviolet CD of DNase. The CD spectrum between 310 to 250 nm is quite complex, and detailed interpretation would be difficult. Nevertheless, the negative and positive bands above 290 nm can be assigned to optically active tryptophans (21). Below 290 nm, the CD bands must be due to overlap of tyrosine and tryptophan bands (21, 22). DNase contains two disulfide bonds whose inherent optical activity undoubtedly contributes to the complexity (23). Despite such difficulty in interpretation, it is clear that calcium causes significant changes in the near ultraviolet CD spectrum. Fig. 6 is the near ultraviolet CD difference spectrum and shows more clearly the maximum differences between DNase with and without calcium. In general, calcium leads to an intensification of the CD bands with major peaks centered at 275 and 284 nm. Low-temperature CD of model tryptophan, tyrosine, and phenylalanine compounds leads to an intensification of the CD bands as a result of increased asymmetry due to restricted rotational and vibrational degrees of freedom (21, 22, 24). If calcium caused the environment of the tryptophan and tyrosine residues in DNase to become more hydrophobic, the result would be restricted degrees of freedom. This would also lead to increased asymmetry giving the observed increased intensity of the aromatic CD bands. Fig. 7 shows that calcium induces a large ultraviolet difference spectrum in DNase. This shift to longer wave lengths in the spectrum is characteristic of the spectral shifts observed when aromatic amino acids are exposed to a more hydrophobic environment (25). Thus, the environment of the tryptophan and the tyrosine residues in DNase is probably becoming more nonpolar when calcium is bound to the enzyme.

**DISCUSSION**

Radioactive 46Ca++ has been used to study the binding of calcium ions to DNase (18). Price has shown that 2 calcium ions bind strongly to DNase at pH 7.5, with an average dissociation constant of $1.4 \times 10^{-4}$ M. At pH 5.5 only 1 calcium ion binds strongly to DNase, with a dissociation constant of $2.2 \times 10^{-4}$ M. The site with high affinity for Ca++ at pH 5.5, and one of the two sites for Ca++ at pH 7.5, is specific for calcium. Even in the presence of a 500-fold excess of magnesium or manganese, a single calcium ion still binds to DNase with a dissociation constant of about $3.2 \times 10^{-4}$ M (18).

It seems probable that the divalent metal ion-binding site responsible for the structural change observed by CD measurements and the protection against trypsin are associated with the calcium-specific site. The dissociation constant of the calcium-specific site, $1.4 \times 10^{-4}$ M, is close to the concentration of calcium which gives half the maximum CD change, $10^{-4}$ M (Table II), and half the maximum protection against trypsin, $1.3 \times 10^{-4}$ M (18). Neither magnesium nor manganese ions bind to the calcium-specific site (18). Neither manganese nor magnesium give the maximum change in the CD spectrum, nor do they
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20. SCHUMAKER, V. (1968) Biochemistry 7, 3427

protect DNase completely from inactivation by trypsin. When magnesium and manganese are present at a 500-fold excess over calcium, calcium can still bind to the calcium-specific site with high affinity (18) and calcium can still give the maximum increase in ellipticity at 215 nm. We therefore suggest that when calcium binds to the calcium-specific site, a conformational change results leading to a more ordered structure of the polypeptide backbone and renders unavailable for reaction the tryptic-sensitive bond or bonds. Since we observed no change in sedimentation coefficient in the presence of calcium, the structural change probably does not involve large alterations in the shape or volume of the protein.

Another hydrolase from the bovine pancreas, trypsin, is also dramatically affected by calcium (26). Changes in ORD and absorption spectra, electrophoretic mobility, stability, and activity were all observed (26). The rate of trypsin-catalyzed activation of trypsinogen is markedly increased by calcium ions (27). These structural effects of calcium ions on both pancreatic trypsin and DNase undoubtedly have physiological significance since both enzymes operate in the intestine where calcium concentrations are high (28) and maximum stability of the proteins would be beneficial.

The spectral changes induced by calcium above 250 nm probably represent different divalent metal ion-binding sites than are associated with the CD changes below 250 nm. Both the CD spectrum above 250 nm (Fig. 5) and the ultraviolet difference spectrum (Fig. 7) have not reached the maximum changes at $10^{-4}$ M calcium and continue to change up to $10^{-3}$ M calcium. The CD spectral changes below 250 nm reach saturation at $10^{-4}$ M calcium. It is possible that the calcium-induced changes between 250 nm and 310 nm are associated with the catalytic active site. Poulos and Price (11) found that N-bromosuccinimide oxidizes a single tryptophan in DNase and abolishes enzymatic activity. Calcium could no longer induce the characteristic difference spectrum in the N-bromosuccinimide-modified enzyme (11). It is possible that the essential tryptophan is in or near the active site. If this same tryptophan is responsible for part of the calcium-induced ultraviolet difference spectrum, then elimination of the calcium-induced difference spectrum suggests that one class of calcium-binding sites is near the essential tryptophan. Divalent metal ions are necessary to alkylate the active site histidine of DNase (10). Half the maximum rate of inactivation is achieved with 0.02 M manganese (10). The strict requirement for the divalent metal ion in the alkylation reaction suggested that the catalytic active site has affinity for divalent metal ion. Therefore, it seems reasonable to suggest that at least one of the weaker divalent ion-binding sites represented by the complex changes in the CD spectrum above 250 nm and the ultraviolet difference spectrum is associated with the catalytic active site.
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