**Cobalt Activation of Carboxypeptidase A**

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In the course of an investigation of the possible role of metal ions in the activity of carboxypeptidase B it was observed that preincubation of this enzyme in the presence of certain metal salts strongly enhanced its peptidase activity. In view of the suggested similarity in mechanism of action of the pancreatic carboxypeptidases (1, 3, 4), the reported instability of partially purified carboxypeptidase B (1), and the background of evidence on the purity, stability, and specificity of carboxypeptidase A (5, 6), it was decided to employ the latter enzyme in primary metal activation studies.

Details are presented here for the simple preincubation procedures employed to effect an increase of percentage in peptidase activity of carboxypeptidase A by the action of cobaltous ions. Studies of the effects of pH, metal ion concentration, and incubation time on this activation phenomenon, as well as specificity and stability of the activated enzyme are reported.

A sensitive and convenient spectrophotometric assay procedure for carboxypeptidase A, similar to that previously outlined for acylase 1 (7) is described.

**EXPERIMENTAL**

**Materials**—The two samples of carboxypeptidase A (Worthington Biochemical Corporation, three times crystallized) employed in these studies were further recrystallized once by the gradient dialysis technique and two additional times by the isoelectric precipitation method (8).

CBZglycyl-L-phenylalanine (Mann Research Laboratories, Lot No. A-5260), employed as a substrate in most of the studies, was found to be completely hydrolyzable to CBZglycine and L-phenylalanine by carboxypeptidase A under suitable conditions.

The solutions of cobaltous salts were prepared from cobaltous acetate .4 H2O or cobaltous chloride .6 H2O, both J. T. Baker Chemical Company c.p. salts.

**Methods**—Peptidase measurements were carried out at 25° in a 0.025 M Tris buffer, pH 7.65, containing 0.1 M NaCl. Stock solutions of carboxypeptidase A in 10% LiCl were prepared fresh daily from well washed enzyme crystals. Enzyme concentrations were calculated from absorption at 278 mμ, assuming a molar extinction coefficient of 8.6 X 10^4.

Solutions of 0.01 M CBZglycyl-L-phenylalanine were employed for assay by the spectrophotometric method unless otherwise stated. Samples of 3 ml of substrate were placed in 1-cm quartz cells in the Beckman model DU spectrophotometer and density set at 232 or 233 mμ (slit width at 0.2, photomultiplier at sensitivity position 4) against a blank of a mixture of 0.01 M each of CBZglycine and L-phenylalanine in the same buffered salt solution used in assays. Enzyme solution was introduced in a volume of from 1 to 10 microliters and readings were taken every minute for a desired length of time. In Fig. 1 are shown curves obtained by plotting concentration of substrate versus optical density difference between substrate and the products of hydrolysis at several wave lengths. In cases where peptide substrates other than CBZglycyl-L-phenylalanine were employed, the colorimetric ninhydrin procedure (9) was used to measure the extent of hydrolysis.

Esterase measurements for carboxypeptidase A were carried out with 0.01 M hippuryl-L-phenylalanic acid (10) as substrate in 0.005 M Tris at pH 7.65 containing 0.1 M NaCl. Measurements were made with the use of a Radiometer type TTT1 Titritator (Radiometer, Copenhagen, Denmark) coupled to an Ole Dich No. 38 recorder (Ole Dich, Instrument Maker, Brondby Strand, Denmark). Under these conditions of assay the initial rate of hydrolysis of ester substrate by carboxypeptidase A approximated that of CBZglycyl-L-phenylalanine, although the orders of hydrolysis are different (10, 11).

**RESULTS**

The effect of the pH of the preincubation mixture on the increase in peptidase activity of carboxypeptidase A, at a 0.01 M cobalt ion concentration, is shown in Fig. 2. The optimum pH for activation at this metal ion concentration appears to be between 7.75 and 8.0 with a rapid decline in activation above pH 8.0 where insoluble metal hydroxide quickly precipitates. A more gradual decline in activation occurs at pH's below the optimum.

From the data plotted in Fig. 3 it is apparent that at a fixed enzyme concentration, the rate as well as the extent of activation of this enzyme by Co++ ions is related to concentration of this metal in the incubation mixture. Data obtained with higher concentrations of enzyme indicate that at a fixed metal concentration the rate and percentage of activation are lower at these higher enzyme concentrations. Information available on the basis of limited studies show that the rate of activation of the enzyme by Co++ ion was facilitated by higher temperatures and retarded by lower temperatures applied during the preincubation period. At 0°, 100% increase in activity with 0.01 M Co++ ion and 2 X 10^-4 mg of protein N per ml was apparent only after 6 hours of incubation, whereas at 37° under identical conditions, practically instantaneous activation occurred.
The data plotted in Fig. 4A show that hydrolysis of CBZglycyl-l-phenylalanine by carboxypeptidase A as well as by the cobalt-activated enzyme, follows first order kinetics over a period of 2 hours under the stated conditions of assay.

The curve in Fig. 4B, constructed from the data of Fig. 4A, shows that upon dilution of the cobalt-activated enzyme solutions, with a buffered salt solution identical to that employed in the hydrolysis assays, the increased activity due to metal ion is rapidly lost.

From these data it would appear that a cobalt-enzyme complex is formed at rates and quantities dependent upon cobalt ion concentration and that this complex, at extremely low metal ion concentrations, is stable in the presence of substrate but rapidly dissociated in the absence of substrate.

It may be noted from the data in Table I that, of the metal ions tested under the stated conditions of preincubation, only Co++ ion showed an activating effect on the peptidase activity of carboxypeptidase A. The significant inhibition of this enzyme by Fe++ ion has been previously reported (5).

The rates of hydrolysis of several other peptide substrates for carboxypeptidase A, including CBZglycyl-l-leucine, CBZ-glycyl-1-glutamyl-l-phenylalanine, and CBBZglycyl-l-tryptophan were measured by the colorimetric ninhydrin procedure with both untreated and Co++-treated enzymes, at a 0.025 M substrate concentration. In each case the relative increase in rate of hydrolysis by the cobalt-treated enzyme over the untreated enzyme was equivalent to that observed with CBZglycyl-L-phenylalanine. An increase of 100% in rate of hydrolysis of each substrate was observed after preincubation of enzyme (4 X 10^-3 mg of enzyme N per ml) with 0.01 M Co++ at pH 8.0 for 15 minutes.

The effect of preincubation of carboxypeptidase A with Co++ ion on the hydrolysis of one of its ester substrates, hippuryl-L-phenyllactic acid, was examined. Under conditions where peptidase activity was significantly enhanced, there was no apparent increase in esterase activity. In these experiments the conditions, as to temperature, approximate ionic strength, pH, enzyme concentration, and approximate initial rates of hydrolysis were duplicated in esterase and peptidase assays.

No activity toward the glycylglycine dipeptidase substrate, glycylglycine, the carboxypeptidase B substrate, hippuryl-L-arginine or the leucine aminopeptidase substrate, L-leucinamide, was observed with the cobalt-treated enzyme.

Dialysis experiments indicate that, under the conditions employed for Co++ ion activation of carboxypeptidase A, cobalt does not displace zinc, which has been shown to be both a structural and functional component of this enzyme and to participate in the mechanism of its catalytic action (2). When a solution...
Effects of preincubation of carboxypeptidase A with various metal ions on hydrolysis of carbobenzoxyglycyl-L-phenylalanine

Values expressed as percentage of activity of enzyme preincubated in the absence of added metal ion.

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>Concentration of metal ion</th>
</tr>
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<tbody>
<tr>
<td>Co²⁺</td>
<td>0.01 M</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>202</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>75</td>
</tr>
<tr>
<td>Fe²⁺</td>
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<tr>
<td>Ca²⁺</td>
<td>97</td>
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</tbody>
</table>

DISCUSSION

The early suggestion that carboxypeptidase A is a metalloenzyme (13) has been supported and extended by evidence that zinc is a structural and functional component of this protein (2). Furthermore, it has been shown that the zinc may be removed from carboxypeptidase A with concomitant loss of activity which may be completely restored by the addition of zinc back to the level of 1 g atom per mole of enzyme protein (14). Substitution, in the metal-free enzyme protein, of metal ions of the first transition period resulted in "a significant restoration of (enzymatic) activity" (14).

The present observations demonstrate that, without displacement of the zinc of carboxypeptidase A, the enzymatic activity of this enzyme toward peptide substrates may be increased as much as 100% through simple preincubation of the enzyme in the presence of cobaltous ion. Since removal of cobalt ions, either by dilution or dialysis, results in reversal of activating influence it would appear that this metal, if it forms a metal-enzyme protein complex, is bound in a manner quite different and much less strongly than is zinc.

Under the present experimental conditions, it is very possible that activation by other metal ions, as well as further activation by Co²⁺ ion, may be masked by a degree of inhibition of enzymatic activity exerted by excess of these metal ions. It is significant in this regard, that preincubation with ferrous ion enhances the peptidase activity of carboxypeptidase B.

Treatment of carboxypeptidase A with Co²⁺ ions does not result in a change in specificity of the enzyme toward peptide substrates. This is borne out by the observed equivalent increase in activity toward different peptide substrates and by the failure of the activated enzyme to hydrolyze peptides which do not conform to the specificity requirements of the parent enzyme.

It has been postulated that the formation of a coordinate bond with metal ion at substrate amide nitrogen is involved in the mechanism of hydrolysis of certain amide substrates by metalloenzymes (6). The fact that an increased rate of hydrolysis of hippurylphenylactic acid was not observed with the cobalt-treated carboxypeptidase A may be related to the inability of cobalt to form a coordinate bond with nitrogen of the substrate.

Fig. 4. Action of cobalt-activated carboxypeptidase A on CBZglycyl-L-phenylalanine after dilution of enzyme-cobalt solutions. A. Points from which the curves on the semilogarithmic plot were constructed, were taken every minute and closely adhered to these curves. The concentration of enzyme N in assay tubes was 3.52 × 10⁻⁸ mg per ml. Diluted enzyme-cobalt solutions contained 1.06 × 10⁻⁸ mg of enzyme N per ml and were 2.5 × 10⁻⁶ M in Co²⁺ ion after preincubation for 3 hours at 25° at a 0.025 M Co²⁺ ion concentration. Numbers in parentheses indicate time in minutes that assays were performed after dilution of enzyme-cobalt solutions with 0.025 M Tris buffer of pH 7.65 containing 0.1 M NaCl. Curve at 115 minutes was identical with that of unactivated enzyme. B. Curve constructed from data of Fig. 4A.
this substrate to form a coordinate bond with metal ion at its ester oxygen.

SUMMARY

As much as 100% increase in the peptidase activity of carboxypeptidase A has been effected by incubation of solutions of this enzyme in the presence of cobaltous ion. No apparent increase in esterase activity accompanies the increased peptidase activity. Preliminary studies of this reaction indicate that the extent of activation of the enzyme is dependent upon pH, metal ion concentration, enzyme concentration, and temperature and time of incubation. The increased activity is rapidly lost in the absence of substrate after dilution or removal of Co⁺⁺ ion by dialysis. The specificity of the enzyme toward several peptide substrates is unchanged by cobalt-activation. Of the metal ions tested under the present incubation conditions, only cobaltous ion showed this enzyme-activating effect. A sensitive and convenient spectrophotometric assay procedure for carboxypeptidase A is outlined and was employed in certain of these studies.

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Addendum—During the course of the preparation of this manuscript, it was brought to our attention by Dr. B. L. Vallee that metal ion activation of carboxypeptidase A, over and above the reported "significant restoration of activity" of the metal-free enzyme protein by ions of the first transitional period (14), has been observed.

REFERENCES

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