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Effects of Metal Salts on the Structure and Activity of α-Chymotrypsin in Ethanol/Water

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The catalytic activity and circular dichroic (CD) spectra of α-chymotrypsin (CT) were measured in ethanol/water (95/5, v/v) solution containing small amounts of metal salts. Although the catalytic activity of CT increased upon the addition of all the metal salts used, the magnitude of activity increase was different for different metal salts. Especially, calcium acetate accelerated the transesterification of amino acid up to 6 fold at 100 μM. The secondary and tertiary structures of CT were also changed by metal salts, as studied by CD measurements. The effects of metal salts on the stability of CT in ethanol/water were also studied, and it was found that the residual activity of CT after 7 days in ethanol/water in the presence of $Ca(OCOCH_3)_2$ was about 20% of the initial activity. The change in activity was closely correlated with the change in the mean residue ellipticity of CT at 208 or 230 nm.

During the past decade, enzyme reactions in organic solvents have been becoming common in applications to organic synthesis and optical resolution.[1] In general, the enzyme activity in an organic solvent is much lower than that in aqueous solutions, and is a complex function of the nature and composition of the organic solvent. Furthermore, recent studies have shown that the activity of enzymes in organic solvents can be greatly increased by organic additives, such as amides or amines.[2—4] However, the effects of metal salts on the enzyme activity in organic solvents have rarely been investigated, except for metalloenzymes.[5] Recently, we found that small amounts of metal salts accelerated the CT-catalyzed hydrolysis and transesterification in organic solutions containing small amounts of water.[6,7] It has been assumed that the activity increase of CT in organic solvents might be ascribed to its structural changes due to specific interactions between CT and metal ions.

Circular dichroism (CD) has been established as a useful technique for detecting various conformational changes of proteins and peptides.[8,9] CD spectra are highly sensitive to the folding or unfolding of peptide chains, and can be used to monitor the changes of secondary and tertiary structures of proteins in solutions. So far, many reports have dealt with CD for detecting the structural changes of enzymes by thermal or chemical denaturing. Changes in the CD spectra of CT in organic/water solutions were also studied with respect to changes in its catalytic activity.[10] In this paper, we report on the relevance of the catalytic activity of CT to its structural changes in ethanol/water (95/5, v/v) in the presence of metal salts.

Results and Discussion

First, the reaction rates of transesterification of *N*-acetyl-L-tyrosine methyl ester (ATME) to *N*-acetyl-L-tyrosine ethyl ester (ATEE) and the hydrolysis of ATME to *N*-acetyl-L-tyrosine (AT) in ethanol/water (95/5, v/v%) were measured in order to investigate the effects of metal ions on the catalytic activity of CT. The results are shown in Fig. 1. Upon the addition of about 10—20 μM of metal salts, the total reaction rate increased for all of the metal salts used. Especially, the addition of $Ca(OCOCH_3)_2$ dramatically increased the reaction rate up to about 6 fold at 100 μM of the metal salt.

A further increase of the metal salt did not significantly increase the reaction rate.

It has been reported that the addition of tertiary amines or formamide accelerated the transesterification of ATME

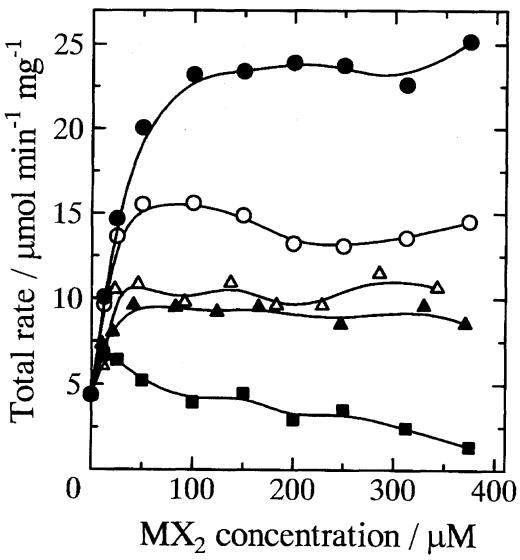

Fig. 1. Effects of metal salts on activity of CT in ethanol/water (95/5, v/v). Total rate is sum of the reaction rates of transesterification and hydrolysis of ATME. Symbols: filled circle, $Ca(OCOCH_3)_2$; open circle, $CaCl_2$; filled triangle, $MgCl_2$; open triangle, $SrCl_2$; filled square, $Ca(H_2PO_4)_2$.

in ethanol/water. However, much larger amounts of these additives were required than metal salts for any measurable acceleration of the reaction; for example, the rate of transesterification was increased by about 6 fold due to 0.2—0.5 M of triethylamine or formamide (1 M = 1 mol dm^{-3}) in ethanol/water.[3,4] These results suggest that metal salts are much more effective than tertiary amines or formamide for accelerating the reaction.

When $CaCl_2$ was used, the maximum reaction rate was obtained at about 50 μM of the salt. Furthermore, $Ca(H_2PO_4)_2$ was less effective to increase the reaction rate, and excess amounts of the salt decreased the reaction rate. These results indicate that the anions of metal salts control the extent of activation of CT. However, a comparison of chlorides of Ca, Mg, and Sr as additives suggests that the nature of metal ions also affects the activity of CT, as can be seen in Fig. 1.

It was recently reported that the catalytic activity of an enzyme in an organic solvent could be altered by the pH value of the aqueous solution from which the enzyme was lyophilized. In general, organic solutions are not buffered, and the above-mentioned results were interpreted as being a "pH memory"[11] of the enzyme. In the present study on the catalytic activity of CT for transesterification, CT was dissolved in an aqueous solution of a metal salt, and the solution was mixed with an ethanol solution of ATME. Therefore, there is a possibility that the pH of aqueous CT solutions containing a metal salt may affect the activity of the enzyme. However, as shown in Table 1, no correlation was found between the relative rate of transesterification and the pH value of the aqueous CT solutions containing metal salts. This result seems to rule out the possibility of activity change due to the pH memory described above.

In organic solutions, it may be reasonably assumed that ionic species have stronger interactions than in aqueous solutions because of the less polar nature of organic solvents than water. Enzymes are globular molecules with ionic or polar groups predominantly on their surface. In organic solutions, the strong interactions between an enzyme and ionic species derived from metal salts would alter the higher structures of the enzyme.

Table 1. Effects of Metal Salts on Transterification of ATME to ATEE in Ethanol/Water Solution Containing Metal Salts[a)]

Metal salt	Ion concn μM	Relative rate of transesterification	pH (of metal salt solution)
Distilled water	—	1.00	—
$Ca(OCOCH_3)_2$	12.5	2.30	10.4
	150	5.41	9.6
	250	5.50	9.2
$Ca(H_2PO_4)_2$	12.5	1.68	5.0
	150	1.10	4.0
$MgCl_2$	41	2.24	9.3
	247	1.98	8.9
$SrCl_2$	11	1.35	8.6
	23	2.47	8.1
	183	2.23	7.1

a) The mixtures of 1 mg of CT, 10 mM of ATME, 9.5 ml of ethanol, 0.5 ml of water, and metal salts were incubated at 30 °C.

Firstly, we attempted to monitor the change in the higher structure of CT by fluorescence spectroscopy. However, there was no detectable change. Thus, in the present work, the structural changes of CT were monitored by CD spectroscopy in both the absence and presence of metal salts. The CD spectra of CT in ethanol/water (95/5, v/v) containing $Ca(OCOCH_3)_2$ are shown in Fig. 2(a) and Fig. 2(b) for the far- and near-UV regions, respectively. For a comparison,

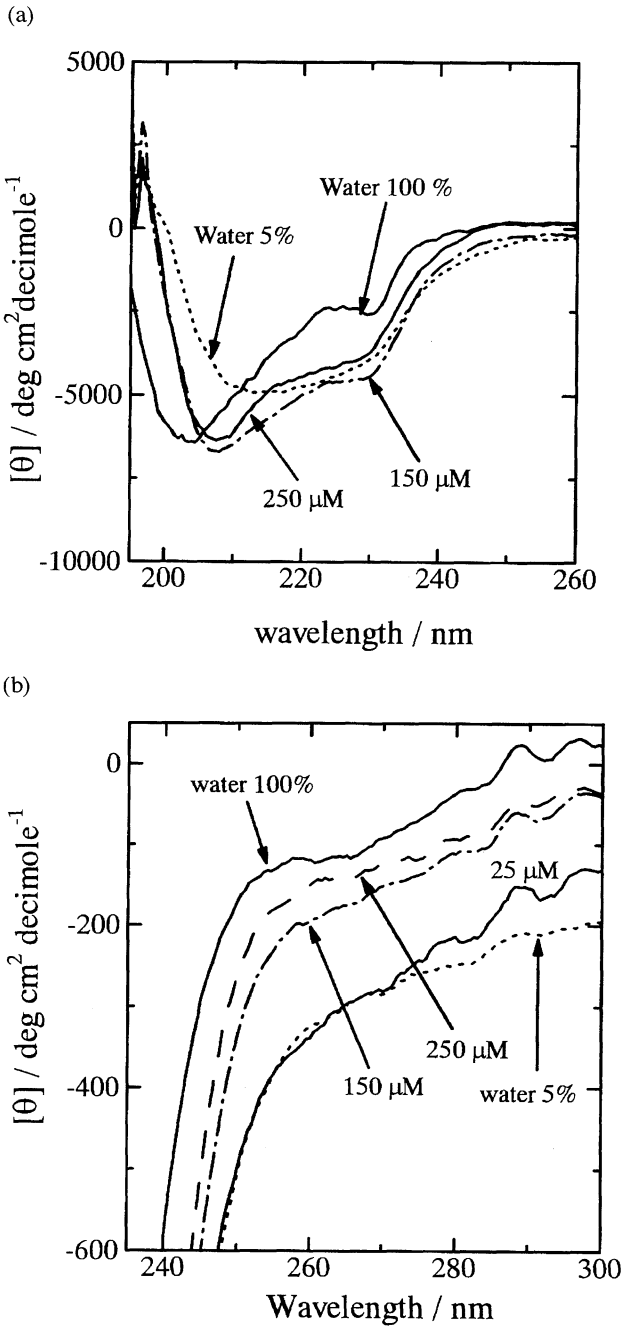

Fig. 2. Far-UV (a) and near-UV (b) CD spectra of CT in water and ethanol/water (95/5, v/v) with or without $Ca(OCOCH_3)_2$.Water contents (v/v%) and $Ca(OCOCH_3)_2$ contents (μM) are shown in the figures.

the spectrum in 100% water is included in Fig. 2, in which CT takes a native conformation and exhibits characteristic negative bands at 203 and 230 nm.[12,13] It can be seen that in ethanol/water (95/5, v/v) without metal salts these bands were not clearly shown, but upon the addition of 150 or 250 μM of $Ca(OCOCH_3)_2$, the CD spectra clearly exhibited large negative bands at 208 and 230 nm. It is known that CD bands in far-UV region (190—260 nm) are due to the secondary structure of proteins. CT is classified to all-β protein on the basis of an X-ray crystallographic analysis:[14,15] The contents of β-sheet and α-helix structures were estimated to be about 34 and 9%, respectively.[16] The CD data in Fig. 2(a) suggest that, without metal salts, a relaxation of the secondary structure occurs in ethanol/water (95/5, v/v), but $Ca(OCOCH_3)_2$ assists CT to retain its native-like secondary structure.

It is known that CD bands of proteins in the near-UV region (260—300 nm) are due to aromatic amino acid residues; a change in the CD band in this region is indicative of a change in the tertiary structure of the protein. Therefore, less-resolved or weak CD bands of CT in ethanol/water (95/5) than those in water (Fig. 2(b)) are considered to reflect changes in the tertiary structure. In contrast, in the same mixed solvent with 25 μM of $Ca(OCOCH_3)_2$, CT exhibited well-resolved CD bands between 280—300 nm, which suggests the recovery of a native-like structure. These results clearly show that the effects of $Ca(OCOCH_3)_2$ on the catalytic activity of CT, as manifested in Fig. 1, is a consequence of the retention of native-like higher structures of the enzyme, which would otherwise be modified by an interaction with ethanol.

As described above, the anions of metal salts also influence the catalytic activity of CT in ethanol/water (95/5). For example, $CaCl_2$ is less effective than $Ca(OCOCH_3)_2$ for accelerating the reaction, as shown in Fig. 1. This can be explained by a smaller effect of the former for maintenance of the higher structure of CT than the latter, as can be seen in Fig. 3(b) and Fig. 4(b). Both in the far- and near-UV regions, the CD bands of CT with $CaCl_2$ are less resolved than those with $Ca(OCOCH_3)_2$. Also, we studied the influence that $MgCl_2$ and $SrCl_2$ exert on the CD of CT (Figs. 3 and 4). The characteristic bands of CT at 208 and 230 nm were not clearly shown, even in the presence of more than 100 μM of these salts. These results, along with the results for $CaCl_2$, suggest that the catalytic activity of CT also depends on the nature of the anions of metal salts. The fact that $Ca(H_2PO_4)_2$ accelerated the reaction only below a limited concentration (about 25 μM) and that the reaction rate gradually decreased at higher concentrations also supports the above consideration (Fig. 1).

CT is not a metalloenzyme, and has no specific sites for metal ions. Therefore, the structural changes of CT caused by metal salts may be a consequence of non-specific interactions between ionogenic groups of the enzyme and metal cations or counter anions. It is known that, in the case of metalloenzymes, structural changes occur by binding specific metal ions to specific sites of the proteins, which is indispensable for the activation of the enzymes.[17—19] However, non-specific binding of ions can also cause structural changes of proteins;

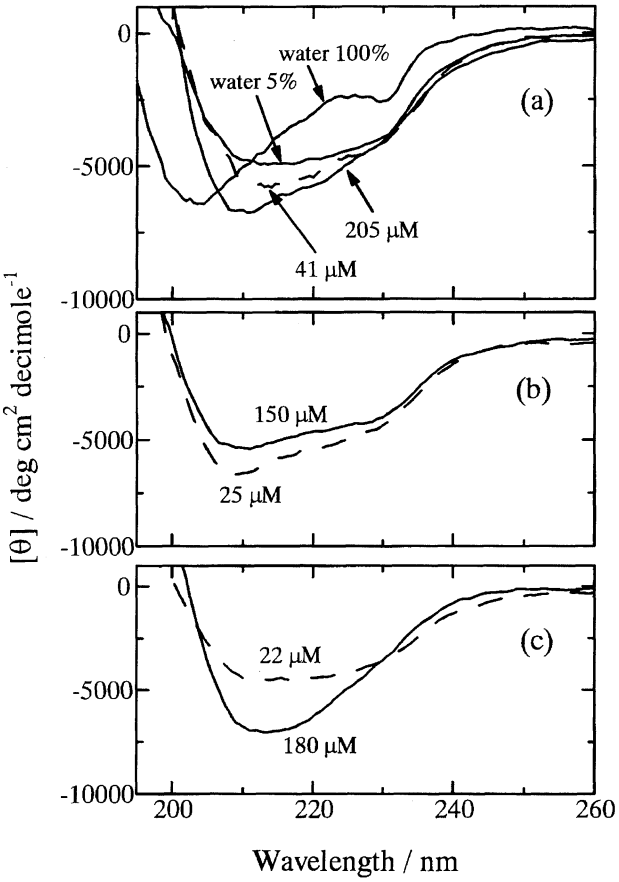

Fig. 3. Far-UV CD spectra of CT in water and ethanol/water (95/5, v/v) containing metal salts. (a) $MgCl_2$; (b) $CaCl_2$; (c) $SrCl_2$. Metal salt contents (μM) are shown in the figure.

an example is the activation of thermolysin by high concentrations (1—5 M) of NaCl in aqueous solutions, which was considered to be due to a modification of the enzyme structure by the shielding surface charges, thus promoting the binding of hydrated ions to the surface of the enzyme, and causing a disintegration of the water structure.[5] In our study, large increases in the activity of CT were obtained at less than 100 μM of metal salts. This may have been due to the low polarity of the media, which would enhance ionic interactions between the enzyme and ionic species derived from metal salts.

As for non-specific effects of metal ions, divalent metal ions seem to be more effective for the retention of the native structure of proteins. In the present study, NaCl did not accelerate CT-catalyzed reactions in ethanol/water (95/5). Brazil et al.[20] demonstrated that the divalent cations (1—10 mM of Ca^{2+}, Mg^{2+}, and Zn^{2+}) significantly changed the secondary structure of the *Escherichia coli* protein GroEL, as detected by the fluorescence spectroscopy of tyrosine residues, whereas monovalent cations (K^+ and Na^+) had little effect. Our results also indicate that divalent cations have stronger interactions with CT than do monovalent ones.

The stability of the higher structure of enzymes is another important feature for applying enzymes in organic solvents. Although a number of reports are available on the thermal

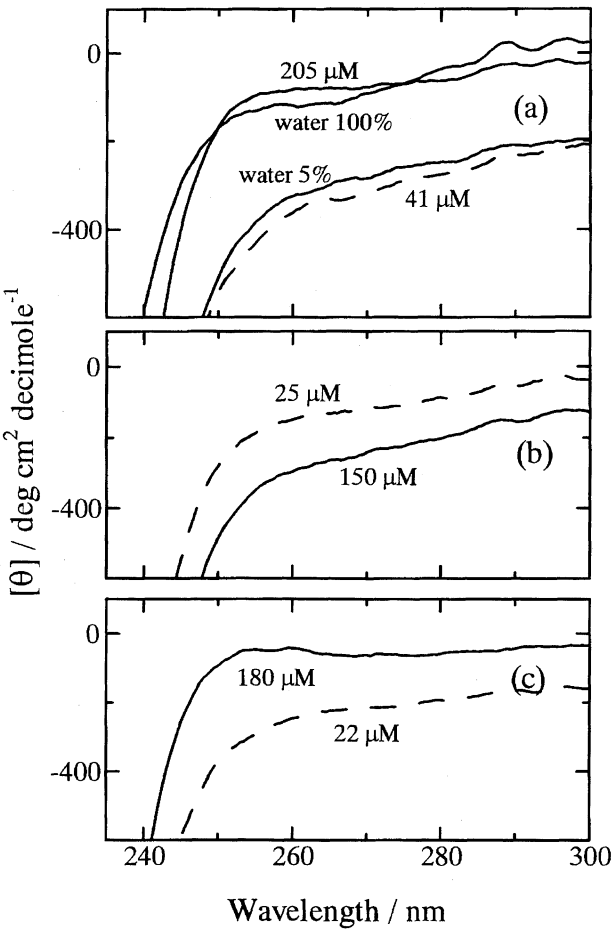

Fig. 4. Near-UV CD spectra of CT in water and ethanol/water (95/5, v/v) containing metal salts. (a) $MgCl_2$; (b) $CaCl_2$; (c) $SrCl_2$. Metal ion contents (μM) are shown in the figure.

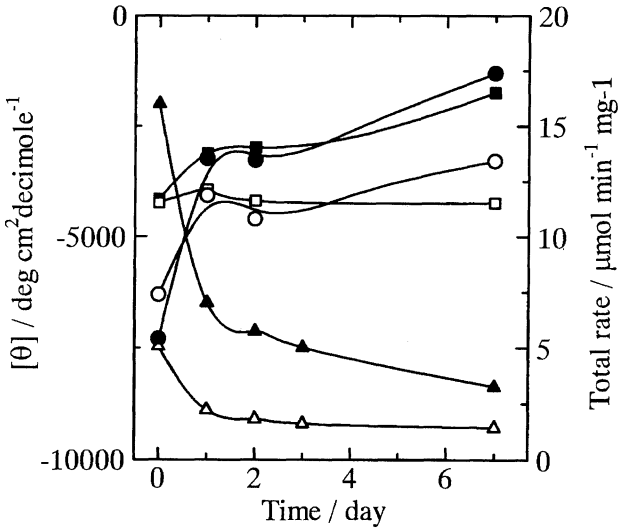

Fig. 5. Stability of CT in ethanol/water (95/5, v/v). Symbols: filled circle, mean residue ellipticity ([θ]) at 208 nm in the presence of $Ca(OCOCH_3)_2$; open circle, [θ] at 208 nm in the absence of Ca $(OCOCH_3)_2$; filled square, [θ] at 230 nm, $Ca(OCOCH_3)_2$; open square, [θ] at 230 nm in the absence of Ca $(OCOCH_3)_2$; filled triangle, total reaction rate in the presence of $Ca(OCOCH_3)_2$; open triangle, total reaction rate in the absence of Ca $(OCOCH_3)_2$.

stability and effects of chemical denaturants, such as urea in water, few reports have appeared on the stability of enzymes in organic solvents. We studied the stability of CT by measuring the CD spectra and the catalytic activity of CT after storage of the enzyme in ethanol/water (95/5) for specified periods of time. The results are shown in Fig. 5. The catalytic activity of CT in ethanol/water (95/5), evaluated by the total reaction rate of ATME (transesterification plus hydrolysis) greatly decreased after storage of the enzyme in the same solvent for 24 h. No significant difference was found for the activities of CT stored in ethanol/water (95/5, v/v) with or without $Ca(OCOCH_3)_2$. Also, the mean residue ellipticity of the bands at 208 and 230 nm exhibited similar changes during storage in ethanol/water (95/5) in both the presence and absence of the metal salt. These results indicate that $Ca(OCOCH_3)_2$ has only small effects on the stability of CT. This means that an enhancement of the catalytic activity of CT by addition of the salt is due to the retention of the native-like structure of CT, which can not be maintained for prolonged periods of time. Based on far- and near-UV CD spectra, it was suggested that, in the absence of metal salts, the secondary structure of CT was transformed instantaneously from the native distorted β-sheet to the regular β-sheet structure. The native structure seems to gradually collapse, even in the presence of metal salt.

In conclusion, the catalytic activity of CT in ethanol/water (95/5) increased significantly upon the addition of divalent metal salts. From a study on the CD spectroscopy of CT, it was suggested that added metal salts contribute to the retention of the native-like structure of the enzyme, probably through ionic or dipolar interactions between the enzyme and the ions derived from the salts. A low activity of enzymes in organic solvents represents a serious limitation to the use of enzyme reactions for preparative purposes. Although metal salts are one of the potential candidates for the activation of enzymes in organic solvents, a more comprehensive study is required with respect to the stability of enzymes under these conditions.

Experimental

α-Chymotrypsin (CT) was purchased from Sigma Chem. Co. (type-II, three- times recrystallized and essentially salt-free). The purity of the enzyme preparation was 85% by spectroscopic active site titration.[21] *N*-Acetyl-L-tyrosine (AT) was purchased from Wako Pure Chem. Ind., Ltd. *N*-Acetyl-L-tyrosine methyl ester (ATME) was prepared by the reaction of AT with methanol in the presence of thionyl chloride. Organic solvents of guaranteed grade were dried on molecular sieves 3A. Metal salts of guaranteed grade were obtained from Wako Pure Chem. Ind., Ltd., and used without further purification.

The transesterification of ATME to *N*-acetyl-L-tyrosine ethyl ester (ATEE) was performed as follows: CT (1 mg) was dissolved in 0.5 ml of an aqueous solution of a metal salt, and the solution was kept standing for 10 min. Then, the solution was added to 9.5 ml of a solution of ATME and acetanilide in ethanol. Acetanilide was used as an internal standard for HPLC analysis. The total volume

of the reaction mixture was 10 ml, and the concentration of ATME was 10 mM. In this paper, the concentration of ATME and metal salts is expressed as the molar concentration based on the total reaction volume. The reaction mixtures were apparently homogeneous (transparent) solutions, and were incubated with constant reciprocal shaking (about 150 cycles per min) at 30 °C. Samples of the reaction mixture were taken at intervals and filtered by poly(tetrafluoroethylene) membrane filters. The filtrate was injected into an HPLC (Shimadzu LC-6A) with a column packed with Shimpack CLC-ODS, which was eluted by water–acetonitrile (50 : 50 by volume). The rates of transesterification and hydrolysis were calculated from the initial increases in the amount of ATEE and AT, respectively. The total rate was represented as the transesterification rate plus the hydrolysis rate. Data were reproducible within about ±10% (at maximum) on repeated runs.

Circular dichroism (CD) measurements were performed on a JASCO 720A spectropolarimeter which was continuously purged with N_2. The temperature was controlled using a water bath which circulated water through a cell holder. Measurements were made at 30 °C for 0.1 mg enzyme/ml solutions using rectangular cells with 1 mm pathlength for the far-UV region and with 10 mm pathlength for the near-UV region. All data were obtained at least two-times measurements, and the average of two or four consecutive scans in far- or near-UV regions, respectively, were taken. CD data are expressed as the mean residue ellipticity $[\theta]$ in deg cm^2 decimole^{-1}.

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