Journal of Inclusion Phenomena and Molecular Recognition in Chemistry **18**: 291–300, 1994.

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Inclusion Complexes of Proteins: Interaction of Cyclodextrins with Peptides Containing Aromatic Amino Acids Studied by Competitive Spectrophotometry*

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(Received: 16 Feburary 1994; in final form: 18 April 1994)

Abstract. The stability constants were measured of inclusion complexes formed from aromatic amino acids and their oligopeptides with α- and β-cyclodextrin, hydroxypropyl β-cyclodextrin, and partially methylated β-cyclodextrin. The method of competitive spectrophotometry with p-nitrophenol as a competing reagent was used, and measurements were made at pH 7.4. β-Cyclodextrin formed complexes of higher stability than the other hosts. The stability of complexes of oligopeptides containing L-phenylalanine was invariably higher than that of L-phenylalanine itself. A model for interaction of proteins with cyclodextrins is proposed, in which the most stable complexes are formed when the native functional form of proteins is unfolded and the nonpolar residues that are buried inside the structure are exposed to water. The complexation of the unfolded structure favors its formation; thus thermal denaturation of proteins is easier in the presence of cyclodextrins. On the other hand, this complexation prevents the intermolecular association of unfolded structures by noncovalent hydrophobic bonding between the exposed nonpolar residues; furthermore, the unfolded complexed forms may revert to the native functional form. This prevention of intermolecular association may explain the stabilizing effect of cyclodextrins on solutions of proteins: a return to the native form is achieved more easily from the complexed, unfolded form than from the unfolded, aggregated forms.

Key words: α-Cyclodextrin, β-cyclodextrin, β-cyclodextrin derivatives, aromatic amino acids, peptides, p-nitrophenol, inclusion complexes, stability constants, competitive spectrophotometry.

1. Introduction

It is well known that cyclodextrins and their derivatives form inclusion complexes with a great variety of compounds [1, 2]. The relatively hydrophobic cavity of cyclodextrins can provide temporary asylum for hydrophobic parts of molecules dissolved in water; this can result in increased solubility and/or solution stability of hydrophobic or amphiphilic compounds. The solubilizing ability of cyclodextrins is currently of interest in the development of pharmaceutical formulations of improved bioavailability [2, 3].

Storage and administration problems are inherent in the rapidly growing group of drugs based on peptides and proteins [4]. Some of the therapeutically useful proteins have limited solubility; others form solutions of limited stability. These

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complications are especially serious for the products of bioengineering. Similar problems, when encountered in drugs of small molecular weight, were alleviated by the formation of inclusion complexes with cyclodextrins. Several findings indicate that when such problems occur with protein/peptide drugs, they also can be alleviated by cyclodextrins. In the presence of hydroxypropyl β-cyclodextrin, the thermal precipitation of porcine growth hormone was reduced [5], the solubility of bovine growth hormone was increased [6]; interleukin-2 became soluble and its aggregation was inhibited [6]; precipitation in bovine insulin solutions was prevented [6]; the stability of several lyophilized protein formulations was enhanced [7, 8]; the solubility of a cyclopeptide antibiotic, gramicidin S, was increased [9]; the β-amyloid protein injection produced no marked short-term toxicity in rat hippocampus, probably through prevention of aggregation [10]. Cyclodextrins, furthermore, were used to reduce the temperature-induced polymerization of β-casein [11]. The efficacy of rectal and nasal administration of insulin in rats and rabbits was increased by cyclodextrin derivatives [12–16].

These findings suggest that inclusion complexation may stabilize the native functional form of proteins in solutions. However, the comparison of the spectral properties of insulin in the presence and the absence of cyclodextrin suggests that the effects of cyclodextrin on protein structures are quite small [16]. Furthermore, differential scanning calorimetry of solutions of several globular proteins revealed a decrease in the transition temperature in the presence of α- and β-CD, an indication that cyclodextrins decrease the thermal stability of globular proteins [17].

The fact that cyclodextrins have stabilizing and destabilizing effects on proteins in solutions illustrates the complexity of the interactions involved and indicates that the use of model compounds with low-molecular weights may be advantageous [18, 19]. Data from the literature indicate that aromatic amino acid residues are primarily responsible for the interaction of proteins/peptides with cyclodextrins [17, 19]. Unfortunately, the existing data [18–23] on complexes between cyclodextrins and aromatic amino acids or their oligopeptides were obtained under varied conditions. Here, to obtain mutually comparable data, we evaluate the formation of complexes between cyclodextrins and aromatic amino acids or oligopeptides obtained by competitive spectrophotometry at physiological pH. We use the data to propose a model for explaining the above mentioned contradictory behavior.

2. Experimental

α-Cyclodextrin and β-cyclodextrin (both from the American Maize Products Company, Hammong) were recrystallized from water and dried under vacuum over phosphorus pentoxide. The hydroxypropyl β-cyclodextrin used was produced by Wacker-Chemie GmbH, Lot 6691. The average degree of substitution was 6.3. The pattern of the substitution on glucose residues, expressed in molar percentages, was as follows: S0: 31.4; S2: 26.8; S3: 7.7; S6: 3.2; S2,3: 16.3; S2,6: 3.7; S3,6: 1.4; S2,3,6: 2.5. Randomly substituted methyl β-cyclodextrin was also a product of

Wacker-Chemie GmbH, Log BW 1/7/92. The average degree of substitution was 12.6. The pattern of substitution on glucose residues, expressed in molar percentages, was as follows: S0: 6.0; S2: 13.6; S3: 5.0; S6: 14.2; S2,3: 6.8; S2,6: 26.9; S3,6: 11.8; S2,3,6: 16.0. Both β-cyclodextrin derivatives were dried under vacuum over phosphorus pentoxide before use. p-Nitrophenol (Aldrich Chemical Company Inc., Milwaukee) was recrystallized from water. L-phenylalanine, aspartame, and oligopeptides were of commercial origin (either Sigma Chemicals, St. Louis or Research Organics Inc., Cleveland) and were used as received.

Measurements of pH were carried out with a Beckman Instruments pHI 43 pH-meter with a combined glass electrode. Phosphate-buffered isotonic saline was prepared from ultrafiltrated deionized water, NaCl (final concentration 0.14 M), and NaH_2PO_4 (final concentration 0.01 M); its pH was adjusted to 7.4 by NaOH.

Spectrophotometric measurements were made on a Beckman Instruments spectrophotometer, Model DU-50. The temperature throughout the experiments was 23 ± 1 °C. Absorbancies were measured at 346 nm; the wavelength corresponded to the isosbestic point of p-nitrophenol.

The apparent stability constants of complexes between cyclodextrins and p-nitrophenol and the apparent differences in molar absorptivities of complexed and free p-nitrophenol were obtained by a non-linear regression. The experimental dependencies of the absorbance of a 0.0003 M p-nitrophenol solution on the cyclodextrin concentration were fitted to the theoretical equations (see Appendix and Figure 1).

Stability constants of complexes between cyclodextrins and amino acid/oligopeptides were determined from absorbance values of solutions of p-nitrophenol and cyclodextrin in the presence of amino acid/oligopeptides. Theoretical equations (see Appendix) were solved numerically for a 1 : 1 stoichiometry model. The required parameters for the cyclodextrin/p-nitrophenol system were obtained as described above.

Because the observed effects were rather small, experiments had to be conducted carefully. Suitable concentrations of reagents were chosen: 0.0003 M for p-nitrophenol, 0.001 for α-cyclodextrin, 0.004 M for β-cyclodextrin and its derivatives, and final concentrations from 0.005 to 0.02 M for amino acid/oligopeptides. A stock solution of p-nitrophenol and cyclodextrin in phosphate-buffered saline was used as a solvent for preparing the solutions of amino acid/oligopeptides. Because the buffering capacity of phosphate-buffered saline is rather low, it was necessary to readjust the pH of amino acid/oligopeptide solutions to 7.4. Four volumes of amino acid/oligopeptide solution subsequently were added to the solution of p-nitrophenol and cyclodextrin in the measuring cell. The absorbance at 346 nm was recorded after the solution was mixed with a magnetic stirrer. The values of absorbance were corrected for the absorption of a guest compound, and stability constants were calculated independently from each measured value. The results are presented as a mean and a standard deviation of the above four measurements.

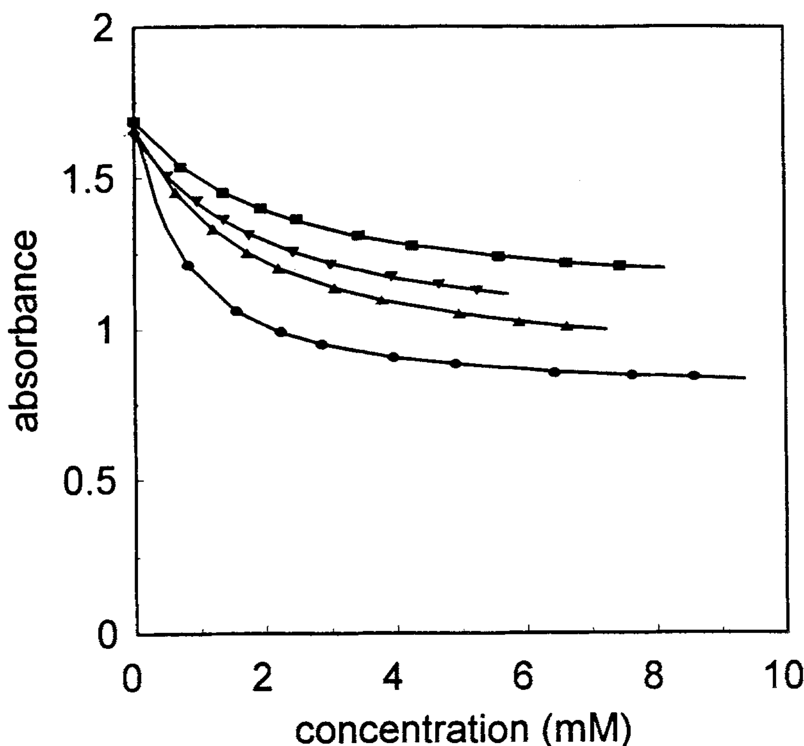

Fig. 1. The dependence of the absorbance of 0.0003 M p-nitrophenol solutions on the concentration of cyclodextrins: α-cyclodextrin (•), β-cyclodextrin (■), hydroxypropyl β-cyclodextrin (▼), and methylated β-cyclodextrin (▲). Phosphate-buffered isotonic saline pH 7.4 was used as a solvent. Curves were obtained by fitting experimental data to the theory.

3. Results

Competitive spectrophotometry is an established method for studying the stability of cyclodextrin inclusion complexes. Until now, however, it has been used mostly at low or high pH [24, 25]. Such pH levels have been used because the competing reagent, usually an acid-base indicator, is present predominantly in one form. As shown in the Appendix, however, an acid-base indicator can be treated as a one-form competing agent at any pH, as long as this pH is kept constant. p-Nitrophenol already has been used as a competing reagent for complexes of α-cyclodextrin at pH 11 [22]. Because p-nitrophenol is known to form complexes with both α-cyclodextrin and β-cyclodextrin at low pH and high pH [24, 26], we selected it as the competing reagent for our experiments at pH 7.4.

Gelb *et al.* [27] were the first to use p-nitrophenol for the determinaton of stability constants of α-cyclodextrin in neutral pH. We depart from their procedure in two important ways. Firstly, we carried out the measurements at the wavelength of an isosbestic point for free p-nitrophenol/p-nitrophenolate rather than in the

TABLE I. Spectrophotometric characterization of complexes between cyclodextrins and p-nitrophenol at pH 7.4 and at wavelength 346 nm.

Cyclodextrin	K_I' (M^{-1})[a]	$\Delta\varepsilon'$ $(M^{-1}\,cm^{-1})$[b]	SD[c]
α-cyclodextrin	1602	−3014	0.0016
β-cyclodextrin	497	−2023	0.0030
hydroxypropyl β-cyclodextrin	531	−2326	0.0036
methylated β-cyclodextrin	619	−2708	0.0011

[a] Apparent stability constant.
[b] Apparent difference in molar absorptivities: the complexed minus the free form.
[c] Standard deviation of residuals in absorbance units.

vicinity of the absorption maximum. This wavelength selection makes the measurement less prone to slight pH variation. Because we used a higher concentration of p-nitrophenol and made measurements at pH above pK_d, the sensitivity of the method was not decreased substantially. Secondly, the values of stability constants were calculated numerically by solving exact equations given in the Appendix rather than by the semiempirical graphical procedure.

Figure 1 and Table I summarize the results of spectrophotometric characterization of complexes between p-nitrophenol and α-cyclodextrin, β-cyclodextrin, randomly methylated β-cyclodextrin, and hydroxypropyl β-cyclodextrin. The apparent stability constants of complexes of α-cyclodextrin and β-cyclodextrin are close to those reported for p-nitrophenolate [24, 26, 27]; this is understandable because pK_D values of 6.90–7.15 for the dissociation constant of p-nitrophenol were reported [28]. Thus p-nitrophenolate should be more abundant at the pH value of 7.4, which we used.

Table II summarizes the results on amino acids and oligopeptides. L-Phenylalanine forms detectable inclusion complexes with all cyclodextrins studied. Their stabilities are relatively low; a slightly higher stability constant was found for β-cyclodextrin than for α-cyclodextrin (17 M^{-1} vs. 13 M^{-1}). The stabilities of L-phenylalanine complexes with randomly methylated β-cyclodextrin and hydroxypropyl β-cyclodextrin were noticeably lower.

The attachment of a glycine residue to the amino group of L-phenylalanine increased the stabilities of all complexes, more for β-cyclodextrin and its derivatives than for α-cyclodextrin. Further extension of the peptide by another glycine residue resulted in additional stabilization of the complexes. When the glycine residue was attached to the carboxyl of L-phenylalanine, the stability of the complex was increased somewhat for the β-cyclodextrin and its derivatives but remained unchanged for α-cyclodextrin.

Our results did not show any enantioselective binding for aromatic oligopeptides. The stabilities of complexes of glycyl-L-phenylalanine and of glycyl-D-phenylalanine were almost identical for all cyclodextrins studied.

TABLE II. Stability constants, K^G, of complexes between cyclodextrins and amino acid/oligopeptides at pH 7.4 measured by competitive spectrophotometry with p-nitrophenol.

Substrate[a]	$K^G \pm$ st.dev. (M^{-1})			
	α-CD	β-CD	MeβCD	HPβCD
L-Phe	13±0.4	17±0.6	4±0.5	5±0.5
Gly-L-Phe	20±0.6	76±1.0	10±0.8	14±0.8
Gly-D-Phe	23±0.7	67±1.2	9±0.7	13±0.5
L-PheGly	14±0.7	57±3.5	16±1.0	22±2.0
GlyGly-L-Phe	47±0.8	89±1.9	11±0.9	21±1.2
Gly-L-Tyr	13±0.2	101±2.0	13±0.6	16±0.9
L-TrpGly	21±1.1	52±1.5	39±1.6	50±3.8
Gly-L-Ala	—[b]	—[b]	—[b]	—[b]
Aspartame	42±0.7	150±3.2	84±5.3	74±4.6

[a] Ala = alanine, Gly = glycine, Phe = phenylalanine, Tyr = tyrosine, Trp = tryptophan, Aspartame = N-L-α-aspartyl-L-phenylalanine methyl ester, α-CD = α-cyclodextrin, β-CD = β-cyclodextrin, MeβCD = partially methylated β-cyclodextrin, HPβCD = hydroxypropyl β-cyclodextrin.
[b] Not detectable.

Under the conditions of our measurements, L-tyrosine and L-tryptophan were not soluble enough to obtain reliable results by the present method. Fortunately, solubilities of two dipeptides containing these amino acids were found to be higher. Cyclodextrin complexes of glycyl-L-tyrosine had stabilities comparable to those of glycyl-L-phenylalanine. An exception was the complex with β-cyclodextrin, which had considerable higher stability.

Analogous comparison of L-tryptophylglycine with L-phenylalanylglycine showed that a substitution of tryptophan for phenylalanine increased the stability of a complex with α-cyclodextrin, had no effect on a complex with β-cyclodextrin, and significantly increased the stabilities of complexes with β-cyclodextrin derivatives.

To eliminate the possibility that the glycine residue also forms inclusion complexes, we conducted the measurements with glycyl-L-alanine but detected no complexation.

The determination of high stability constants for aspartame (i.e. N-L-α-aspartyl-L-phenylalanine methyl ester) indicates that the elimination of charges in the vicinity of an aromatic group increases the stability of an inclusion complex. The findings of Goldberg and Rekharsky on other peptide-type guests [29] and the results of a comparison of aliphatic amino acids with alcohols [30] lead to similar conclusions.

Some of the complexes characterized in Table II, including the complex between α-cyclodextrin and L-phenylalanine [18–23], have been studied previously. Most

of the published values of the stability constant of this complex are in the range 10–16 M^{-1}, even though a variety of pH and experimental methods were used [18, 19–22]. The value of 13 M^{-1} which we found, falls in the middle of this range. The stability constants of aspartame complexes were also measured by calorimetry at pH 4 [31]. The published value, 128 M^{-1}, agrees closely with our result, 150 M^{-1}. The difference is somewhat higher for hydroxypropyl β-cyclodextrin: 46 M^{-1} by calorimetry, 74 M^{-1} in this study. This result is not surprising because hydroxypropyl β-cyclodextrin is not a chemical individuum; thus somewhat different behavior may be expected for different samples. Almost identical stability constants were reported for α-cyclodextrin complexes with glycylglycyl-L-phenylalanine, L-phenylalanylglycylglycine, and L-phenylalanine [18]. This finding agrees with our results for L-phenylalanylglycine but differs in regard to oligopeptides with L-phenylalanine on the C-terminus. Note that the direct spectrophotometry used in Ref. [18] can detect only complexation that alters the absorption spectrum of an aromatic group. The competitive spectrophotometry used in the present study is not limited in this way.

4. Discussion

In the native conformation of globular proteins, the hydrophobic groups are buried in the interior of the molecule. In that way they avoid unfavorable contacts with water [32] and interactions with cyclodextrins. Indeed, cyclodextrins have minimal effects on the conformation of proteins in solutions [16]. The native conformation of proteins, however, is not static; partial reversible unfolding occurs, whose frequency and extent increase with temperature. As the transition temperature is approached, the protein may start unfolding into conformations in which some of the hydrophobic groups are exposed [32]. The energies of these unfolded conformations can be reduced by including the hydrophobic groups in cyclodextrin. Thus the transition of a protein from native to unfolded states may occur at lower temperatures, as previously observed [17].

The complexation of cyclodextrins with the temporarily exposed hydrophobic groups may also explain the stabilizing effect of cyclodextrins on solutions of proteins. Aggregation of proteins probably begins with the clustering of the temporarily exposed hydrophobic groups, which occurs in an intermolecular fashion. In other words, protein molecules lose their solubility through crosslinking by noncovalent bonding, which occurs between the exposed residues of hydrophobic amino acids on different molecules. Inclusion of such exposed groups in cavities of cyclodextrin may prevent them from interacting with one another and may increase the opportunity for a partially unfolded protein to fold back into its native form, in which the hydrophobic groups are not exposed to the solvent.

The above model explains the observations made previously about proteins, which are described in the introduction and are supported as well by the present results on oligopeptides. The oligopeptides used here may be regarded as a model

for the unfolded protein structures. The present results show that oligopeptides containing hydrophobic amino acids form complexes with cyclodextrins of the same stability as the amino acids themselves or of higher stability. That finding was not fully expected. The peptide backbone represents a bulky substituent, which through steric strains, may prevent formation of inclusion complexes. These backbone-caused steric strains obviously are not dominant in the interaction of an oligopeptide with cyclodextrin; thus they probably do not play a role in the interaction of unfolded conformations of globular proteins with cyclodextrins. The increase in the strength of interaction that occurs when an amino acid is incorporated into an oligopeptide may be due to possible hydrogen bonding between secondary hydroxyls of cyclodextrin with the peptide backbone and to a removal of ionic or zwitterionic species from the immediate vicinity of the cavity.

Acknowledgements

The authors thank Drs. G. Schmid and T. Wimmer of Wacker Chemie Co. for donating the fully characterized β-cyclodextrin derivatives used in this study and to the unknown referee for bringing Ref. [27] to their attention.

Appendix: Competitive Spectrophotometry at pH $\simeq$ pK_d

The theory of competitive spectrophotometry is quite well established [24, 25, 27]. If an acid-base indicator is used as the competing reagent and if the pH of the solution is about equal to the pK_D of the indicator, then both acidic and basic forms are present at similar concentrations and must be taken into account. We will show that even under such conditions, the theory of the one-form competing reagent can be used for data treatment as long as the pH is held constant during the experiment.

Let us suppose that only 1 : 1 complexes are formed between cyclodextrin and the guest compound and between cyclodextrin and the competing reagent. Furthermore, let us suppose that the absorption at the wavelength used is the sum of the absorptions of bound and free competing reagents. If we take into account the expressions of mass balance and the definitions of stability constants, the absorbance A of the solution of a one-form competing reagent in the presence of cyclodextrin and the guest compound is expressed by

$$A = A_0 + \frac{\Delta\varepsilon\, K_I C_I [\mathrm{CD}]}{1 + K_I[\mathrm{CD}]} \tag{A1}$$

$$C_{CD} = [\mathrm{CD}]\left(1 + \frac{K_I C_I}{1 + K_I[\mathrm{CD}]} + \frac{K_G C_G}{1 + K_G[\mathrm{CD}]}\right) \tag{A2}$$

where C_{CD}, C_I, and C_G are the total molar concentrations of cyclodextrin, the competing reagent, and the guest compound respectively; [CD] is the molar concentration of free cyclodextrin; K_I and K_G are the stability constants of complexes

between cyclodextrin and a competing reagent and between cyclodextrin and a guest compound; A_0 is the absorbance of a solution of the same C_I, but $C_{CD} = 0$; $\Delta\varepsilon$ is the difference between molar absorptivities of bound and free competing reagents. Equation (A1) states that the change in the absorption upon the complexation is given by the change in the molar absorptivity caused by the complexation and by the concentration of the complex formed. If K_I and $\Delta\varepsilon$ are known, the value of [CD] can be calculated from Equation (A1) and the value of K_G from Equation (A2). Values of K_I and $\Delta\varepsilon$ can be determined from the experiment in the absence of a guest compound.

If the competing reagent is an acid-base indicator, then the ratio of concentrations of free acidic and basic forms is determined by pH and the dissociation constant K_D. If both acidic and basic forms of the competing reagent are able to form a complex with cyclodextrin, Equations (A3) and (A4) hold true rather than Equations (A1) and (A2).

$$A = A_0 + \frac{\left(\Delta\varepsilon_{IH} K_{IH} \frac{[H^+]}{K_D} + \Delta\varepsilon_{I^-} K_{I^-}\right) C_I [CD]}{1 + \frac{[H^+]}{K_D} + [CD]\left(K_{IH} \frac{[H^+]}{K_D} + K_{I^-}\right)} \tag{A3}$$

$$C_{CD} = [CD]\left(1 + \frac{C_I\left(K_{IH} \frac{[H^+]}{K_D} + K_I\right)}{1 + \frac{[H^+]}{K_D} + [CD]\left(K_{IH} \frac{[H^+]}{K_D} + K_{I^-}\right)} + \frac{K_G C_G}{[CD]\, K_G}\right). \tag{A4}$$

Indexes IH and I^- denote quantities related to the acidic and basic forms of a competing reagent. Equations (A3) and (A4) can be written in the form of Equations (A1) and (A2) if an apparent stability constant, K_I', and an apparent difference in molar absorptivities, $\Delta\varepsilon'$, are defined as follows:

$$K_I' = \frac{K_{IH} \frac{[H^+]}{K_D} + K_{I^-}}{1 + \frac{[H^+]}{K_D}} \tag{A5}$$

$$\Delta\varepsilon' = \frac{\Delta\varepsilon_{IH} K_{IH} \frac{[H^+]}{K_D} + \Delta\varepsilon_{I^-} K_{I^-}}{K_{IH} \frac{[H^+]}{K_D} + K_{I^-}} \tag{A6}$$

This means that the acid-base indicator can be treated as a one-form competing reagent if the pH is kept constant during the experiment and if the apparent values of K_I and $\Delta\varepsilon$, determined at the same pH, are used. In principle, the value of A_0 also

depends on pH for the acid-base indicator; therefore it should be treated similarly to K_I and $\Delta\varepsilon$. This situation can be avoided, however, if the measurements are made at the wavelength corresponding to the isosbestic point in the absorption spectrum of a free competing reagent.

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