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FTIR determination of ligand-induced secondary and tertiary structural changes in bovine plasminogen

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Human plasminogen undergoes a large tertiary structural change in the presence of lysine derivatives (e.g. ε-amino caproic acid, EACA). This change facilitates human plasminogen activation by human plasminogen activators, resulting in elevated blood plasmin levels. It is hypothesized that this structure-function relationship is similar for bovine plasminogen. The objectives of this study were to investigate the effect of the ligand EACA on the secondary structure of plasminogen (bovine, human, and rabbit) and the tertiary structure of bovine plasminogen using Fourier-transform infrared spectroscopy (FTIR). Spectra of plasminogen, EACA, and a mixture of plasminogen and EACA in water and deuterium were collected using FTIR. Fourier-self deconvoluted spectra in the amide I region (1700–1600 cm^{-1}) were used to detect changes in secondary structure of plasminogen after EACA addition. Change in bovine plasminogen tertiary structure was determined by comparing ratios of amide II (1600–1500 cm^{-1}) to amide I bond intensities over time for samples in deuterium. No differences in secondary structure were observed for any plasminogen in the presence of EACA; however, addition of EACA significantly changed tertiary structure of bovine plasminogen. This tertiary structural change indicates a transition from a folded to an unfolded state, which could be more easily converted to plasmin. These results are consistent with reported human plasminogen studies using neutron scattering (tertiary structure) and circular dichroism (secondary structure) methods.

Keywords: Plasminogen, plasmin, FTIR, plasminogen structure.

Plasminogen is the zymogen form of plasmin, a native serine proteinase found in milk. Plasminogen concentrations in fresh milk are much higher (2 to 100 times) than plasmin concentrations, and plasminogen is converted to plasmin by the specific activity of plasminogen activators, also naturally present in milk (Deharveng & Nielsen, 1991; Heegard et al. 1994; White et al. 1995; Bastian & Brown, 1996). The presence of plasmin inhibitors and plasminogen activator inhibitors in milk has been demonstrated, but these inhibitors can be inactivated by mild heat treatment and/or by lowering the pH (Precetti et al. 1997). Factors that contribute to the amount of active plasmin in cheese milk are important because plasmin contributes to proteolysis and flavour development in certain cheeses during ripening (e.g. Swiss) but also can adversely affect the quality of other dairy products, as seen in age gelation of ultra-high temperature (UHT) milk (Kohlmann et al. 1991; Fox & McSweeney, 1996).

Many researchers have suggested that bovine plasminogen is associated with casein micelles in milk (Reimerdes & Klostermeyer, 1974; Richardson, 1983; Richardson & Elston, 1984). Baer et al. (1994) showed that lysine-binding as well as electrostatic forces were involved in plasminogen binding to casein micelles. Haissat et al. (1994) found that each type of casein protein has two plasminogen binding sites with different dissociation constants. In model systems, the conversion of plasminogen to plasmin catalyzed by urokinase-type plasminogen activator increased in the presence of native milk proteins (Politis et al. 1995a, b). The specific reason for this increase in activation has not yet been identified; however, once identified this mechanism could be used to control the plasmin system in milk and dairy products.

Researchers have also shown that once β-lactoglobulin, a whey protein, has received a heat treatment it can bind to the surface of casein micelles (Hill, 1989; Dalgleish, 1990; Swaisgood, 1996). Corredig & Dalgleish (1999) found that heat treatment of both major types of whey proteins (α-lactalbumin and β-lactoglobulin) can allow them to bind to κ-casein. The binding of whey proteins to casein micelles might interfere with plasminogen binding to the same casein micelles. This interference in turn may

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have implications on plasminogen activation by reducing the interaction between plasminogen and casein proteins, thereby decreasing plasminogen activation.

Plasmin and plasminogen components of the bovine milk plasmin system are essentially identical to these components in the human blood plasmin system (Schaller et al. 1985; Bastian & Brown, 1996). The primary structure of human plasminogen is a simple polypeptide chain with 790 amino acids (Wallen & Wiman, 1972). This polypeptide has a folding pattern that includes five triple-loop structures (i.e. kringles) and the protease domain (Sottrup-Jensen et al. 1978). There are five lysine-binding sites on plasminogen, and the lysine-binding site contained in kringle one has a dissociation constant of 9 μM, while the other four sites have dissociation constants of 5 mM (Markus et al. 1978). It is thought that plasminogen adheres to casein micelles through these lysine-binding sites.

The activation of human plasminogen, catalyzed by plasminogen activators, is enhanced in the presence of lysine derivatives (Machovich & Owen, 1993). Human plasminogen undergoes a large tertiary structure change (determined by small-angle neutron scattering) in the presence of 50 mM ε-amino caproic acid (EACA, a lysine derivative) while the secondary structure remains unaltered (Mangel et al. 1990). Mangel et al. (1990) concluded that the change in human plasminogen from the closed form (difficult to activate) to the open form (10-fold increase in activation rate) did not require a change in the secondary structure (determined using CD spectroscopy). Secondary and tertiary structural changes in bovine plasminogen in the presence of EACA are unknown.

Fourier-transform infrared (FTIR) spectroscopy has become a powerful method to elucidate the secondary structure of proteins (Curley et al. 1998; Lefevre & Subirade, 1999; Farrell et al. 2001). Global secondary structures of various proteins determined using FTIR spectroscopy were consistent with X-ray crystallography results (Kumosinski & Unruh, 1996). An advantage of FTIR is that it allows the determination of secondary structure of proteins in a wide range of environments, unlike other commonly used techniques such as X-ray crystallography and NMR spectroscopy (Haris & Chapman, 1996; Kumosinski & Unruh, 1996; Griebenow et al. 1999). Early FTIR studies were performed in solid state or deuterium to avoid the interference of water absorption with the absorption of protein in the amide I region. However, recent developments in equipment design and accurate water vapour subtraction techniques enable accurate protein measurements in a more realistic biological environment, water (Curley et al. 1998). FTIR methods are rapid, non-destructive, and are also advantageous in terms of equipment cost (much less than neutron scattering and NMR equipment). Infrared spectra of a protein provide information about protein backbone conformation, side chain structure, and environment (Barth & Zscherp, 2000). Good correlations between protein structure and amide band frequency were obtained from experimental studies and normal-mode calculations (Haris & Chapman, 1996). There are various amide mode vibrations, amide I and amide II. Absorption in the amide I band (1700–1600 cm^{-1}) arises mainly from C=O stretching and is used for secondary structure determination. Absorption in the amide II band (1600–1500 cm^{-1}) is the result of N-H bending vibrations (de Jongh et al. 1996).

FTIR has also been used to obtain information on tertiary structure stability of proteins (Goormaghtigh et al. 1994; de Jongh et al. 1995; Scheirlinckx et al. 2001). FTIR spectra of proteins in deuterium are used for estimation of slowly and rapidly exchanging amide protons. Exchange rates provide information on solvent accessibility and hydrogen bond stability of amide protons (Haris & Chapman, 1996). A large shift of about 100 cm^{-1} occurs in the amide II band upon exposure to deuterium, while the amide I band shifts only 10–15 cm^{-1} (de Jongh et al. 1996). Exchange rates and tertiary structure stability can be followed by monitoring the changes in the amide II band intensity over time for proteins in deuterium.

The objectives of this study were to investigate the effect of the ligand, EACA, on the secondary structure of plasminogen from various sources (bovine, human, and rabbit) and the tertiary structure of bovine plasminogen using FTIR.

Materials and Methods

Sample preparation

Plasminogen from bovine, human, and rabbit plasma, EACA, and deuterium were purchased from Sigma Chemical Co. (St. Louis, MO). Plasminogen samples were dissolved in distilled water to prepare a final plasminogen concentration of 0·00138 g/ml, calculated using the conversion factors provided by Sigma. The total solids concentrations were 0·026 g/ml for bovine, 0·069 g/ml for human, and 0·024 g/ml for rabbit plasminogen due to the different conversion factors. To investigate the effect of the ligand, EACA, on the structure of the plasminogen, EACA (0·00656 g/ml) was added to the plasminogen solutions. The same solutions were prepared in deuterium for tertiary structure determination of bovine plasminogen. Since deuterium is very hygroscopic, the headspace of the small glass vials in which solutions were prepared was flushed with nitrogen immediately after the addition of deuterium, a separate vial was prepared for each measurement, and all vials were stored in a nitrogen-flushed glove box. A separate plasminogen solution and a deuterium control also were prepared for each time interval. Samples were removed from vials with a syringe at 0, 1, 2, 3, 3·5, and 4 h, and immediately placed on an ATR crystal to collect FTIR spectra.

Infrared measurements

Spectra of plasminogen solutions in the absence and presence of EACA, water, deuterium, and ligand (EACA)

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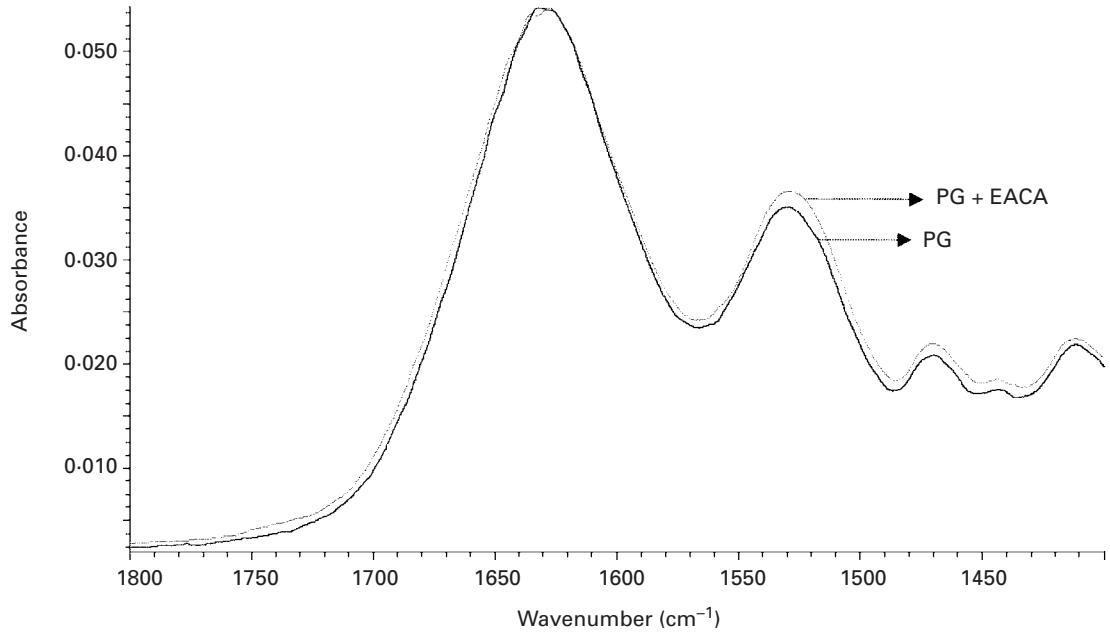


Fig. 1. FTIR spectra of bovine plasminogen (PG) and bovine plasminogen+ε-amino-caproic acid (EACA) in water used for secondary structure analysis.

were obtained using an FTIR spectrometer (ThermoNicolet 670, Madison, WI) equipped with a ZnSe ATR cell with 45° aperture angle and a liquid nitrogen cooled MCTA detector. Equipment was purged with liquid nitrogen during measurements. Samples were prepared and stored for the desired amount of time (0, 1, 2, 3, 3·5, and 4 h) prior to placement on the ATR crystal for data collection. For each sample, 256 scans were recorded at a resolution of 4 cm^{-1} The total time required for data collection was 4 min per sample.

Secondary structure determination

While each native protein spectrum was obtained by subtracting the water spectrum from the plasminogen spectrum, the effect of EACA on plasminogen structure was determined by subtracting the EACA spectrum from the mixture of EACA+plasminogen spectrum (called ligand-added protein spectrum). The subtraction factor was based on the total solids concentration for each sample. Subtracted spectra were used to calculate deconvoluted spectra using PeakFit software (v. 4.1.1, SPSS Inc., Chicago, IL). Curves were fitted assuming a Gaussian shape for resolved components. Full-width at half-maxima (FWHM) was 6·75 and kept constant for all peaks during deconvolution. The relative areas of the component bands, which serve to estimate the fraction of the various secondary structures, were calculated from deconvoluted spectra. Peak assignments for the amide I region were made using the results of Curley et al. (1998). To determine the accuracy of the curve fitting procedure, residual plots, correlation coefficients, and fit standard error were performed. All residuals plots had random appearances. High correlation coefficients of R^2>0·88 were obtained, and fit standard errors were low.

Tertiary structure determination

For tertiary structure determination, deuterium and EACA spectra were subtracted from native protein and EACA-added protein spectra, respectively, for each deuteration time. The areas under the amide I and amide II regions were calculated using TQ software (v. 6.1.1, ThermoNicolet, Madison, WI). The amide II region was normalized to the amide I region to eliminate the possible fluctuations in intensity (de Jongh et al. 1995). A 100% value corresponds to measurements taken at ∼0 h (there was a short unavoidable time delay of less than 5 min due to sample preparation). Obtained ratios of amide II to amide I were expressed as fractions of non-exchanged protons.

Results and Discussion

Secondary structure

In the first part of this study, secondary structures of bovine, human, and rabbit plasminogen with and without the addition of EACA were determined using FTIR spectroscopy. Plasminogen spectra were obtained by subtracting the water and EACA spectra in proportion to the total solid concentration of protein solution according to a modified approach of Rahmelow & Hubner (1997). Fig. 1 shows the infrared spectra of bovine plasminogen

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Table 1. Secondary structure of plasminogen from various sources in the presence (+) and absence (–) of 50 mM-EACA (ε-aminocaproic acid) as determined using FTIR

Values are means of at least three measurements ± SD

Source	β-Sheet, %	Irregular, %	α-Helix, %	Large loop, %	β-Turn, %	Other, %
Bovine (–)	33·2 ± 4·9	9·3 ± 0·7	8·6 ± 0·8	6·9 ± 1·2	17·5 ± 2·7	24·5 ± 7·9
Bovine (+)	38·0 ± 2·8	9·6 ± 0·9	8·3 ± 0·8	6·6 ± 1·1	16·0 ± 4·8	21·5 ± 8·0
Human (–)	37·7 ± 1·5	9·6 ± 0·7	8·6 ± 0·5	7·3 ± 0·3	20·0 ± 1·9	16·9 ± 2·0
Human (+)	40·0 ± 2·3	10·3 ± 0·5	7·8 ± 0·3	6·6 ± 0·3	19·2 ± 1·7	15·9 ± 1·4
Rabbit (–)	43·8 ± 3·7	13·5 ± 5·3	8·1 ± 0·4	5·8 ± 0·4	12·7 ± 2·3	16·2 ± 1·5
Rabbit (+)	42·5 ± 0·4	7·6 ± 0·4	7·7 ± 0·8	6·2 ± 0·1	10·7 ± 0·3	25·0 ± 0·0

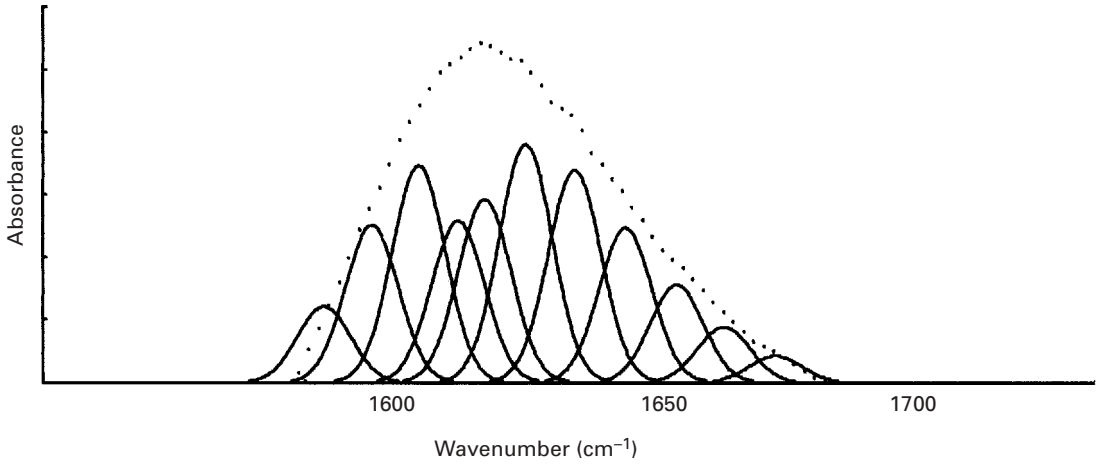


Fig. 2. Deconvoluted FTIR spectra of bovine plasminogen in water used for assigning secondary structures.

in the presence and absence of EACA after the subtraction of water spectra. These spectra consist of the amide I region from 1700–1600 cm^{-1} and the amide II region from 1600–1500 cm^{-1}. Human and rabbit plasminogen spectra were also very similar (data not shown). The deconvoluted form of the spectra was used to elucidate the changes in the secondary structure of bovine plasminogen. Second derivative and deconvolution procedures narrow the widths of IR bands, and bands revealed using these techniques allow the identification of different protein structures. Non-linear curve-fitting analysis of deconvolved amide bands provides information about the quantitative secondary structure. Several studies successfully used this approach to determine the secondary structure of proteins (Byler & Susi, 1986; Kumosinski & Unruh, 1996). Fig. 2 displays the deconvolved spectra of bovine plasminogen. The peak assignments for the amide I region were made using the results of Curley et al. (1998). Specifically, the assignments were: β-sheet = 1640–1620 cm^{-1}, irregular = 1648–1642 cm^{-1}, α-helix = 1652–1648 cm^{-1}, large loop = 1660–1652 cm^{-1}, and β-turn = 1700–1660 cm^{-1} (Curley et al. 1998).

A statistical *t* test performed using data in Table 1 showed that addition of EACA did not have a significant effect on the secondary structure of bovine, human or rabbit plasminogen. Also, no significant shifts in individual peaks for α-helix, β-sheet, or other individual secondary structures were observed across three replicates when EACA was added to plasminogen. It may be argued that the presence of lysine in plasminogen samples purchased from Sigma may have masked the effect additional EACA might have had on secondary structure. However, our observations are consistent with another study that investigated the effect of EACA on human plasminogen secondary structure using circular dichroism (CD) spectroscopy (Mangel et al. 1990). In our study, α-helix content of human plasminogen was more than 2 fold of that determined by CD spectroscopy. However, this discrepancy between CD and FTIR results was also observed in another study (Farrell et al. 2001). Secondary structure of all plasminogen types was similar; however, rabbit plasminogen had the lowest amount of β-turn and human plasminogen had the highest amount of β-turn.

Tertiary structure

The second part of this research investigated the effect of EACA on the tertiary structure of bovine plasminogen. Exchange rates of amide hydrogen for deuterium depend on the stability of protein secondary structure and the accessibility of N-H groups to solvent. For example, proteins having a random structure exchange hydrogen for deuterium

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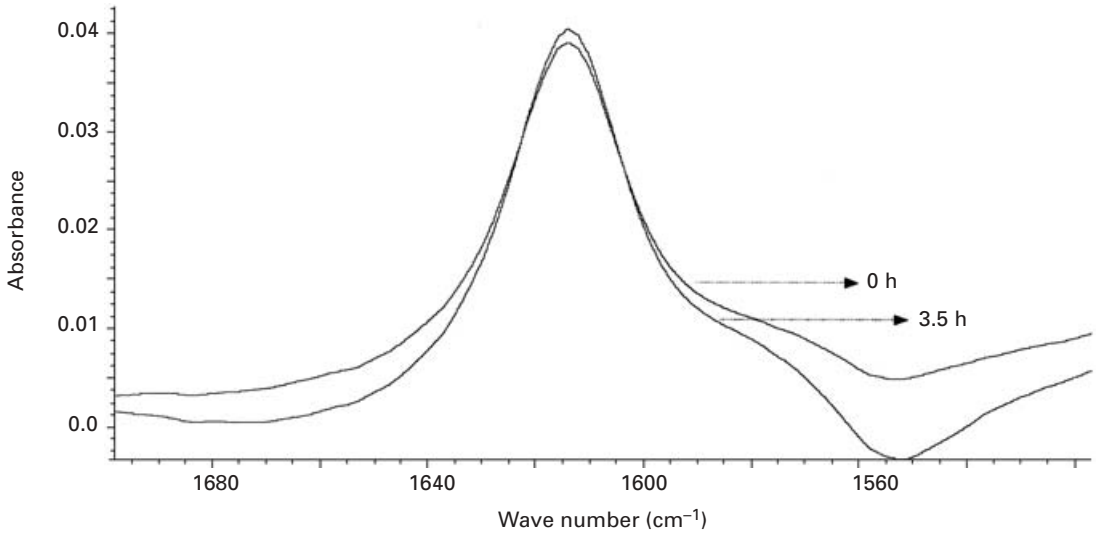


Fig. 3. FTIR spectra of bovine plasminogen+ε-amino-caproic acid (EACA) in deuterium oxide at 0 and 3·5 hours.

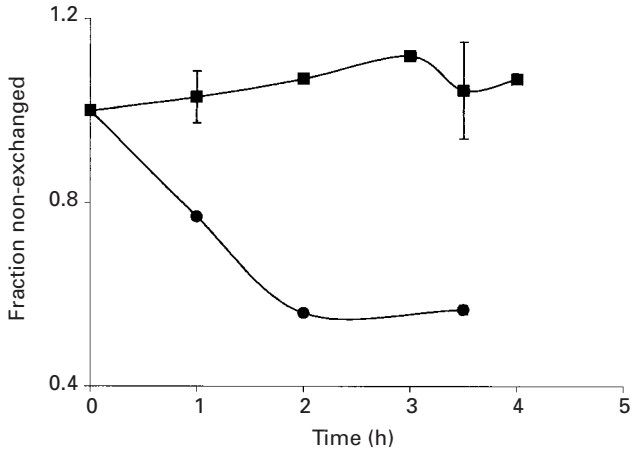


Fig. 4. Fraction of hydrogen ions not exchanged for deuterium during a 3·5 hour incubation of ■, plasminogen or ●, plasminogen+ε-amino-caproic acid (EACA) in deuterium oxide. Significant differences exist (with 95% confidence) where error bars do not overlap (in many cases the error bars are obscured by the plotted points).

more easily than proteins having a regular structure (Scheirlinckx et al. 2001). Therefore, changes in tertiary structure can be followed with the determination of hydrogen/deuterium exchange rates. For this purpose, spectra of plasminogen in deuterium with and without the addition of EACA were obtained using FTIR. The amide I band shifted about 10 cm^{-1}, while the amide II band intensity around 1550 cm^{-1} decreased and shifted to 1450 cm^{-1} (amide II′) with deuteration. Spectra in Fig. 3 show a decrease in the absorbance in the 1500 to 1595 cm^{-1} amide II region with increased exposure to deuterium. A polypeptide that exists in a more extended conformation will have fewer protons non-exchanged or a lower absorbance in the amide II region.

Data in Fig. 4 show the intensity of the amide II band normalized to the amide I band. This ratio is defined as the fraction non-exchanged and is expected to remain around one for tightly folded proteins, following a rapid initial exchange. We believe the 5-min time delay in our data collection prevented detection of this initial exchange; however, comparisons between samples should not be affected by this since all samples had the same time delay. Data in Fig. 4 indicate very clearly that the exchange is greater in the presence of EACA because the fraction non-exchanged decreased. This increased exchange means that a conformational change occurred in bovine plasminogen in the presence of EACA that resulted in a more extended protein structure. The data recorded in Table 1 and the data displayed in Fig. 4 taken together show that bovine plasminogen in the presence of EACA undergoes a large tertiary structural change with only a slight secondary structural change. These data are consistent with the conformational change model proposed by Mangel et al. (1990) for human plasminogen in the presence of EACA using neutron scattering for tertiary structure and CD spectroscopy for secondary structure investigation. That model included a conformational transformation from a closed form to an open form without a change in secondary structure, consistent with our findings using FTIR.

Conclusions

Secondary structure of bovine, human, and rabbit plasminogen measured using FTIR did not change significantly in the presence of EACA. FTIR data indicate that a large change in the tertiary structure of bovine plasminogen occurs in the presence of EACA. This change in structure, caused by lysine-binding, may have implications on the activation of bovine plasminogen.

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