β -Oxidation of polyunsaturated fatty acids with conjugated double bonds

Mitochondrial metabolism of octa-2,4,6-trienoic acid

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The mitochondrial β -oxidation of octa-2,4,6-trienoic acid was studied with the aim of elucidating the degradation of unsaturated fatty acids with conjugated double bonds. Octa-2,4,6-trienoic acid was found to be a respiratory substrate of coupled rat liver mitochondria, but not of rat heart mitochondria. Octa-2,4,6-trienoyl-CoA, the product of the inner-mitochondrial activation of the acid, was chemically synthesized and its degradation by purified enzymes of β -oxidation was studied spectrophotometrically and by use of h.p.l.c. This compound is a substrate of NADPH-dependent 2,4-dienoyl-CoA reductase or 4-enoyl-CoA reductase (EC 1.3.1.34), which facilitates its further β -oxidation. The product obtained after the NADPH-dependent reduction of octa-2,4,6-trienoyl-CoA and one round of β -oxidation was hex-4-enoyl-CoA, which can be completely degraded via β -oxidation. It is concluded that polyunsaturated fatty acids with two conjugated double bonds extending from even-numbered carbon atoms can be completely degraded via β -oxidation because their presumed 2,4,6-trienoyl-CoA intermediates are substrates of 2,4-dienoyl-CoA reductase.

INTRODUCTION

The β -oxidation of unsaturated fatty acids has been the subject of several studies over the past 10 years (for a recent review see ref. [1]). The major outcome of these studies is a revision of the pathway by which unsaturated fatty acids are degraded [1]. Specifically, it is now generally accepted that double bonds extending from even-numbered carbon atoms are reductively removed after they have moved to the 4 position by chain shortening of the fatty acyl-CoA and after they have become part of a 2-*trans*,4-*cis*-dienoyl-CoA system owing to the introduction of a 2-*trans* double bond catalysed by medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) [2]. The reduction of 2-*trans*,4-*cis*-dienoyl-CoA thioesters to 3-*trans*-enoyl-CoAs by NADPH is catalysed by the enzyme 2,4-dienoyl-CoA reductase (EC 1.3.1.34) [3], which has been identified in all tissues and organelles capable of β -oxidation [1].

Fatty acids with conjugated double bonds are naturally occurring compounds which are mostly found as components of triacylglycerols in seed oils [4]. However, fatty acids with conjugated double bonds can also be formed during the autoxidation of lipids [5] and during the partial hydrogenation of vegetable oils [6]. Those fatty acids having conjugated double bonds at even-numbered carbon atoms might yield 2,4,6-trienoyl-CoA thioesters as a result of chain shortening and the introduction of a double bond at the 2 position.

The question addressed in this study is whether a compound like octa-2,4,6-trienoic acid or its CoA derivative can be further degraded by β -oxidation, and if so, by which sequence of reactions? We demonstrate here that octa-2,4,6-trienoic acid is a respiratory substrate of rat liver mitochondria and that octa-2,4,6-trienoyl-CoA is a substrate of the NADPH-dependent 2,4-dienoyl-CoA reductase, which facilitates the further β -oxidation of this compound.

MATERIALS AND METHODS

Materials

NADPH, NADPH⁺, NAD⁺, NADH, acetyl-CoA and agarose–hexane–adenosine 3',5'-bisphosphate were purchased from Pharmacia. Sigma was the source of CoASH, ADP, L-3-hydroxyacyl-CoA dehydrogenase, malate dehydrogenase, citrate synthase, lactate dehydrogenase, acyl-CoA oxidase, catalase and all standard biochemicals. Octa-all-*trans*-2,4,6-trienoic acid and deca-2-*trans*,4-*trans*-dienal were bought from Aldrich Chemical Co. The fatty acid oxidation complex from *Escherichia coli* was purified as previously described [7].

Synthesis of octa-2,4,6-trienoyl-CoA, deca-2,4-dienoyl-CoA and dec-3-*cis*-enoyl-CoA

Commercially available all-*trans*-2,4,6-octatrienoic acid was purified immediately before use by extraction with hot hexane and crystallization at –20 °C. After recrystallization from toluene/methanol, the compound was obtained in the form of white needles with a melting

Fig. 1. U.v. spectrum of octa-2,4,6-trienoyl-CoA

point of 190–193 °C (literature m.p. 189 °C [8]). Deca-2-*trans*,4-*trans*-dienoic acid was prepared from deca-2-*trans*,4-*trans*-dienal by oxidation with Ag₂O [9], and dec-3-*cis*-enoic acid was synthesized from oct-3-yn-1-ol as described [10]. The CoA derivatives of deca-2,4-*trans*-dienoic acid, octa-2,4,6-trienoic acid and dec-3-*cis*-enoic acid were synthesized by the mixed-anhydride method of Goldman & Vagelos [11]. Concentrations of the resulting thioester solutions were determined by the method of Ellman [12] after cleaving the thioester bond with 1 M-hydroxylamine (pH 7). The u.v. spectrum of octa-2,4,6-trienoyl-CoA after purification by h.p.l.c. exhibited an absorbance maximum at 337 nm with an absorption coefficient of 49 300 M⁻¹·cm⁻¹. The absorption coefficient at 340 nm was 48 780 M⁻¹·cm⁻¹ (see Fig. 1).

Isolation of mitochondria and respiration measurements

Rat heart mitochondria were isolated by the procedure of Chappell & Hansford [13]. Rat liver mitochondria were prepared by a similar procedure, except that 0.25 M-sucrose was used instead of 0.21 M-mannitol plus 0.07 M-sucrose and the treatment with Nagarse was omitted. Protein concentrations of mitochondrial suspensions were determined by the biuret method [14].

Rat liver mitochondria (2 mg of protein) or rat heart mitochondria (1 mg of protein) were suspended in 1.9 ml of a basal iso-osmotic medium containing 0.1 M-KCl, 20 mM-Tris/HCl (pH 7.4), 4 mM-potassium phosphate, 4 mM-MgCl₂ and 0.1 mM-EGTA. To this suspension were added, in the indicated sequence, bovine serum albumin (0.5 mg/ml), 0.5 mM-L-malate, 1 mM-ADP, and, 1 min later, one of the substrates. Respiration rates were measured polarographically by use of a Clark oxygen electrode attached to a Gilson oxygraph at 25 °C.

Preparation of a soluble extract of rat liver mitochondria and partial purification of 2,4-dienoyl-CoA reductase

Rat liver mitochondria (50 mg) suspended in 7 ml of 25 mM-potassium phosphate (pH 7.2) containing 0.25 mM-dithiothreitol and 0.5 mM-EDTA were sonicated for 6 × 6 s with a Branson sonifier (model W-185) equipped with a micro-tip, and centrifuged at 105 000 g for 1 h. For the purpose of partially purifying 2,4-dienoyl-CoA reductase, mitochondria were first precipitated by centrifugation at 5900 g for 8 min and resuspended in 50 mM-potassium phosphate (pH 7.2) containing 0.5 mM-dithiothreitol and 1 mM-EDTA. The resulting mitochondrial suspension was sonicated as described above and centrifuged at 105 000 g for 1 h. Protein concentrations of the mitochondrial extracts were determined either by the biuret procedure [14] or by the method of Lowry *et al.* [15].

The soluble extract (30 ml, containing 180 mg of protein) obtained from mitochondria (420 mg of protein) was applied to an agarose-hexane-adenosine 3',5'-bisphosphate column (1 cm × 5 cm) equilibrated with 50 mM-potassium phosphate (pH 7.2) containing 0.5 mM-dithiothreitol and 1 mM-EDTA. The column was washed with 10 column vol. of the same buffer and was developed with a linear NADP⁺ gradient made up of 200 ml each of the starting buffer with and without 1.2 mM-NADP⁺. Fractions were assayed for 2,4-dienoyl-CoA reductase with 2-*trans*,4-*trans*-decadienoyl-CoA as a substrate, and for thioesterase with octa-2,4,6-trienoyl-CoA as substrate. Fractions with high reductase but negligible

thioesterase activities were pooled and concentrated in an Amicon concentrator (PM 10 membrane).

Enzyme assays and enzymic determination of the product formed from octa-2,4,6-trienoyl-CoA by 2,4-dienoyl-CoA reductase

All assays were performed on a Gilford recording spectrophotometer (model 250) at 25 °C. 2,4-Dienoyl-CoA reductase was assayed as described in principle by Kunau & Dommes [3]. The spectrophotometric assay is based on measuring the decrease in *A*₃₄₀. A standard assay mixture (1 ml) contained 0.2 M-potassium phosphate (pH 8), 0.1 mM-NADPH, 30 μM-octa-2,4,6-trienoyl-CoA or 30 μM-deca-2,4-dienoyl-CoA and an amount of partially purified rat liver 2,4-dienoyl-CoA reductase to give an *A*₃₄₀ change of approx. 0.08/min. The thioesterase assay was based on measuring the *A*₃₄₀ decrease owing to the hydrolysis of octa-2,4,6-trienoyl-CoA at pH 8. A standard assay mixture (1 ml) contained 0.2 M-potassium phosphate (pH 8), 30 μM-octa-2,4,6-trienoyl-CoA and enzyme to give an *A*₃₄₀ change of approx. 0.06/min.

After completion of the NADPH-dependent reduction of octa-2,4,6-trienoyl-CoA catalysed by 2,4-dienoyl-CoA reductase, the assay mixture was passed through an Amicon membrane (PM 5) to remove proteins. To the resultant filtrate were added NAD⁺ and CoASH (final concns. 1.2 mM and 0.25 mM respectively), followed by *E. coli* fatty acid oxidation complex (12 munits/ml). Difference spectra of this mixture versus a control, containing all components except the enzyme, were recorded between 280 and 400 nm. When the absorbance ceased to change, L-malate and malate dehydrogenase were added to final concentrations of 3.2 mM and 50 units/ml respectively. The conversion of acetyl-CoA into citrate was initiated by addition of citrate synthase (2 munits/ml) after the malate dehydrogenase reaction had reached an equilibrium. When the *A*₃₄₀ did not increase any further, pyruvate (6 mM) and lactate dehydrogenase (6 units/ml) were added to demonstrate that the absorbance peak centred at 340 nm was due to NADH.

The product formed from octa-2,4,6-trienoyl-CoA after one cycle of β-oxidation was generated by incubating 30 μM of the substrate in 0.2 M-potassium phosphate (pH 8) with 120 μM-NADPH, 0.5 mM-NAD⁺ and 0.1 mM-CoASH in the presence of 2,4-dienoyl-CoA reductase (0.78 munit/ml) and *E. coli* fatty acid-oxidation complex (12 munits/ml). After reaching an equilibrium, the enzymes were removed by ultrafiltration (Amicon membrane YM 5). To the filtrate were added catalase (2 μg/ml) and acyl-CoA oxidase (0.1 unit/ml), and the *A*₃₀₀ was recorded. When it reached a maximum, 60 μM-NADPH and 2,4-dienoyl-CoA reductase (0.78 munit/ml) were added and the *A*₃₀₀ was recorded.

The activity of the *E. coli* fatty acid-oxidation complex is defined by the activity of its component enzyme Δ³-*cis*-Δ²-*trans*-enoyl-CoA isomerase (EC 5.3.3.8) assayed with dec-3-*cis*-enoyl-CoA at pH 8 and 25 °C [7]. A unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate into product per min.

Product analysis by h.p.l.c.

A Waters h.p.l.c. system was used, consisting of two model 6000A pumps, a model 660 solvent programmer,

a model U6K sample injector and a model 441 absorbance detector attached to a Houston Omniscrite chart recorder. A Waters μ Bondapak C_{18} column (30 cm $\times$ 3.9 mm) was placed on-line and equilibrated with 50 mM-ammonium phosphate (pH 5.5). Samples were injected and eluted at a flow rate of 1 ml/min with a methanol gradient (detailed in Fig. 4) containing 50 mM-ammonium phosphate (pH 5.5). The eluted substrates, products and coenzymes were detected at 254 nm with a full-scale recorder setting of 0.2 A unit. All solvents and samples were clarified by filtration through 0.45 μ m-pore-size HA Millipore filters.

RESULTS

Octa-2,4,6-trienoic acid as a respiratory substrate of mitochondria

Coupled rat liver mitochondria, which respire in the presence of exogenously added octanoic acid, ADP and phosphate, also utilize octa-2,4,6-trienoic acid as a respiratory substrate (see Figs. 2a and 2b). However, the rate of respiration observed with octa-2,4,6-trienoic acid was only 30 ng-atoms of O/min per mg of protein. This value represents 40 % of the rate of 72 ng-atoms/min per mg observed with octanoic acid as a substrate. The rate of respiration when determined as a function of the concentration of octa-2,4,6-trienoic acid approached a maximal value of 35 ng-atoms of O/min per mg of

Fig. 2. Rates of mitochondrial respiration: a–d, with rat liver mitochondria; e and f, with rat heart mitochondria

Abbreviations: C_8 , octanoic acid; $[2,4,6]C_8$, octa-2,4,6-trienoic acid; DNP, 2,4-dinitrophenol. Numbers give the respiration rates in ng-atoms of O/min per mg of protein. For experimental details see the Materials and methods section.

protein at a substrate concentration of 30 μ M (results not shown). Neither octanoic acid nor octa-2,4,6-trienoic acid supports respiration in uncoupled rat liver mitochondria (see Figs. 2c and 2d), presumably owing to a lack of intramitochondrial ATP required for the conversion of these medium-chain fatty acids into their CoA thioesters, which are the substrates of β -oxidation and thus of respiration.

In contrast with its ready metabolism in rat liver mitochondria, octa-2,4,6-trienoic acid did not support respiration in coupled rat heart mitochondria, even though octanoic acid served as a respiratory substrate (see Figs. 2e and 2f). This inability of rat heart mitochondria to utilize octa-2,4,6-trienoic acid is most likely a consequence of the relatively narrow substrate specificity of the heart mitochondrial medium-chain acyl-CoA synthetase [16], which must activate the substrate before it can be oxidized.

Reduction of octa-2,4,6-trienoyl-CoA by 2,4-dienoyl-CoA reductase and its subsequent β -oxidation

When octa-2,4,6-trienoyl-CoA was incubated with NADPH and 2,4-dienoyl-CoA reductase, its charac-

Fig. 3. Spectral changes associated with the reduction of octa-2,4,6-trienoyl-CoA and its subsequent β -oxidation

(a) Absorbance changes recorded when octa-2,4,6-trienoyl-CoA was incubated with NADPH and 2,4-dienoyl-CoA reductase for (1) 0 min, (2) 2 min, (3) 12 min, (4) 70 min. (b) Absorbance changes recorded when the reduction product formed as described in (a) was incubated with the *E. coli* fatty acid oxidation complex plus CoA and NAD^+ for (0) 0 min, (1) 6 min, (2) 10 min. The reference cuvette contained all components except for the fatty acid oxidation complex. Further absorbance changes were recorded (3) 5 min and (4) 8 min after the addition of L-malate, malate dehydrogenase, and citrate synthase to the mixture that gave rise to spectrum 2. When lactate dehydrogenase and pyruvate were added to the sample that yielded spectrum 4, recording 5 was obtained. For experimental details see the Materials and methods section.

Fig. 4. H.p.l.c. analysis of the products formed during the reduction of octa-2,4,6-trienoyl-CoA and its subsequent β -oxidation

(a) Products identified when octa-2,4,6-trienoyl-CoA was incubated for 86 min with NADPH and 2,4-dienoyl-CoA reductase.

(b) Products identified 12 min after the *E. coli* fatty acid oxidation complex, CoA and NAD⁺ were added to the reaction mixture containing the products shown in panel (a). Peaks labelled NADP⁺, NADPH, NAD⁺, NADH, CoA, AcCoA (acetyl-CoA) and 2,4,6C₈CoA (octa-2,4,6-trienoyl-CoA) were identified by use of authentic compounds. Samples were eluted with a methanol gradient detailed below the absorbance tracings on both chromatograms.

teristic spectrum with a maximum at 337 nm disappeared in a time-dependent manner (see Fig. 3a). The observed absorbance change was dependent on the presence of both NADPH and the reductase. The apparent kinetic

constants ($V_{\max}$ and K_m) of 2,4-dienoyl-CoA reductase were determined with both deca-2,4-dienoyl-CoA and octa-2,4,6-trienoyl-CoA as substrates at a fixed concentration of 60 μ M-NADPH. The apparent $V_{\max}$ of the

reduction of octa-2,4,6-trienoyl-CoA (90 nmol/min per mg of protein) was less than one-tenth of the rate of 980 nmol/min per mg obtained with deca-2,4-dienoyl-CoA. The apparent K_m value for octa-2,4,6-trienoyl-CoA was 50 μ M, and thus 5 times the K_m value of 10 μ M determined for deca-2,4-dienoyl-CoA.

After establishing that octa-2,4,6-trienoyl-CoA can be reduced by NADPH-dependent 2,4-dienoyl-CoA reductase, the further β -oxidation of the reduction product was studied. For this purpose a reaction mixture obtained after the complete reduction of octa-2,4,6-trienoyl-CoA was freed of protein by ultrafiltration and incubated with purified *E. coli* fatty acid-oxidation complex in the presence of NAD⁺ and CoASH. Since the complex contains all β -oxidation enzymes except for acyl-CoA dehydrogenase and 2,4-dienoyl-CoA reductase, it should β -oxidize the product of the reduction as long as the latter compound contains an isolated double bond in the 2,3-position or 3,4-position. As documented in Fig. 3(b), an increase in the A_{340} was observed, and was attributed to the formation of NADH formed during β -oxidation. Since completion of a cycle of β -oxidation would also yield acetyl-CoA, its presence was tested for and confirmed by a further increase in A_{340} when L-malate, malate dehydrogenase and citrate synthase were added to the reaction mixture (see Fig. 3b). The molar proportions of octa-2,4,6-trienoyl-CoA reduced:NADH formed during β -oxidation:acetyl-CoA and malate into citrate were close to 1:1:1. The absorbance increases centred around 340 nm were shown to be due to the formation of NADH, because the absorbance disappeared when pyruvate and lactate dehydrogenase were added to the reaction mixture (see Fig. 3b).

More direct evidence for the reduction of octa-2,4,6-trienoyl-CoA and its subsequent β -oxidation was obtained by h.p.l.c. analysis. Incubation of octa-2,4,6-trienoyl-CoA, NADPH and 2,4-dienoyl-CoA reductase resulted in the formation of NADP⁺ and compound X, which was eluted from a reverse-phase h.p.l.c. column ahead of octa-2,4,6-trienoyl-CoA (see Fig. 4a). When the *E. coli* fatty acid oxidation complex, NAD⁺ and CoASH were added to the above reaction mixture, β -oxidation occurred, as evidenced by the appearance of NADH and acetyl-CoA (see Fig. 4b). The peak for octa-2,4,6-trienoyl-CoA disappeared almost completely, whereas peak X increased in size (see Fig. 4b). It remains unclear, however, why the product of octa-2,4,6-trienoyl-CoA reduction and the product of its reduction plus β -oxidation seem to emerge at the same position marked 'X'.

Structure of the product obtained after reduction and β -oxidation of octa-2,4,6-trienoyl-CoA

Since the reduction of octa-2,4,6-trienoyl-CoA followed by one cycle of β -oxidation should result in the removal of two double bonds and one acetate unit, the product was expected to be hexenoyl-CoA, most likely with a double bond in the 4,5-position. If so, introduction of a 2,3-double bond should yield hexa-2,4-dienoyl-CoA (sorboyl-CoA), which is a substrate of 2,4-dienoyl-CoA reductase [17]. This prediction was shown to be correct when the product obtained after reduction and β -oxidation of octa-2,4,6-trienoyl-CoA was converted by acyl-CoA oxidase and catalase into a compound with an absorbance at 300 nm characteristic of a 2,4-dienoyl-

Fig. 5. Absorbance changes observed when the product of the reduction and β -oxidation of octa-2,4,6-trienoyl-CoA first reacted with acyl-CoA oxidase and catalase and then with 2,4-dienoyl-CoA reductase and NADPH

Acyl-CoA oxidase and catalase were added at zero time. 2,4-Dienoyl-CoA reductase and NADPH were added at the time marked by the arrow. For experimental details see the Materials and methods section.

CoA chromophore (see Fig. 5). Since addition of NADPH and 2,4-dienoyl-CoA reductase to this presumed 2,4-dienoyl-CoA-containing reaction mixture led to the disappearance of the absorbance at 300 nm (see Fig. 5), the absorbing compound was most likely sorboyl-CoA. Hence the product formed as a result of the reduction and β -oxidation of octa-2,4,6-trienoyl-CoA must have been hex-4-enoyl-CoA.

DISCUSSION

The question addressed in this study was whether fatty acids with conjugated double bonds extending from even-numbered carbon atoms can be degraded via the reductase-dependent pathway of unsaturated fatty acid oxidation. Chain-shortening of such fatty acids should lead to the formation of 4,6-dienoyl-CoA intermediates, which upon dehydrogenation by acyl-CoA dehydrogenase would yield 2,4,6-trienoyl-CoAs. If 2,4-dienoyl-CoA reductase catalyses the NADPH-dependent reduction of 2,4,6-trienoyl-CoA thioesters, this conjugated system of four double bonds would be completely disrupted at a substantial cost in free energy. If the reductase does not act on 2,4,6-trienoyl-CoA thioesters, fatty acids with conjugated double bonds are either degraded by another pathway, or are not at all, or not completely, degraded, with possible deleterious consequences to the animal.

The initial observation that coupled rat liver mitochondria respire with octa-2,4,6-trienoic acid as a substrate proved this compound to be metabolized in liver mitochondria. Presented in Fig. 6 is the sequence of steps by which octa-2,4,6-trienoyl-CoA is most likely degraded. The key question is the reduction of octa-2,4,6-trienoyl-CoA (I) by NADPH, catalysed by 2,4-dienoyl-CoA reductase. If the mechanism of this reductive step is identical with the reduction of 2,4-dienoyl-CoA compounds, octa-3,6-dienoyl-CoA (II) would be formed. The latter compound is then converted into octa-2,6-dienoyl-CoA (III) by Δ^3 -cis- Δ^2 -trans-enoyl-CoA

Fig. 6. Proposed degradation of octa-2,4,6-trienoyl-CoA by β -oxidation

isomerase, which also acts on compounds with 3-*trans* double bonds, although at a slower rate [10]. The subsequent β -oxidation involving the hydration of octa-2,6-dienoyl-CoA (III), followed by the dehydrogenation of L-3-hydroxyoct-6-enoyl-CoA (IV) and the thiolitic cleavage of 3-oxo-oct-6-enoyl-CoA (V), can occur, as evidenced by the formation of NADH and acetyl-CoA, which were detected spectrophotometrically and by h.p.l.c. If this sequence of reaction did occur, then hex-6-enoyl-CoA (VI) should have been formed. The following evidence supports the predicted formation of hex-6-enoyl-CoA. The reaction of presumed hex-6-enoyl-CoA with oxygen in the presence of acyl-CoA oxidase and catalyse gave rise to a compound with an absorbance maximum at 300 nm, as expected if hexa-2,4-dienoyl-CoA (sorboyl-CoA, VII) were formed. Since this chromophore disappeared upon addition of NADPH

and 2,4-dienoyl-CoA reductase, the compound with the chromophore at 300 nm was sorboyl-CoA and its precursor must have been hex-6-enoyl-CoA.

The degradation of octa-2,4,6-trienoic acid in mitochondria requires its initial conversion into octa-2,4,6-trienoyl-CoA, most likely catalysed by medium-chain acyl-CoA synthetase. The subsequent sequence of reaction is outlined in Fig. 6. The key reaction is the NADPH-dependent reduction of octa-2,4,6-trienoyl-CoA by 2,4-dienoyl-CoA reductase. The subsequent reactions are catalysed by the mitochondrial β -oxidation enzymes, all of which are associated with different proteins. The conversion of hex-4-enoyl-CoA (V) into hexa-2,4-dienoyl-CoA (VI) is presumed to be catalysed by medium-chain acyl-CoA dehydrogenase, which is the only one of the mitochondrial acyl-CoA dehydrogenases capable of introducing a double bond between the thioester group and an existing double bond in the 4,5-position [2]. The resultant hexa-2,4-dienoyl-CoA (sorboyl-CoA) is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to yield hex-3-enoyl-CoA (VIII). The complete conversion of hex-3-enoyl-CoA (VIII) into 3 mol of acetyl-CoA requires a shift of the 3,4-double bond to the 2,3-position catalysed by enoyl-CoA isomerase, followed by two cycles of β -oxidation.

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