Mechanistic studies of morphine dehydrogenase and stabilization against covalent inactivation

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Morphine dehydrogenase (MDH) of *Pseudomonas putida* M10 catalyses the NADP⁺-dependent oxidation of morphine and codeine to morphinone and codeinone. This enzyme forms the basis of a sensitive detection and assay method for heroin metabolites and a biotransformation process for production of hydromorphone and hydrocodone. To improve these processes we have undertaken a thorough examination of the kinetic mechanism of MDH. Sequence comparisons indicated that MDH belongs within the aldose reductase enzyme family. MDH was shown to be specific for the pro-R hydrogen of NADPH. In steady-state kinetic studies, product inhibition patterns suggested that MDH follows a Theorell–Chance mechanism for codeinone reduction at pH 7, and a non-Theorell–Chance sequential

ordered mechanism for codeine oxidation at pH 9.5. Residues corresponding to the catalytically important Tyr-48, Lys-77 and Asp-43 of aldose reductase were modified by site-directed mutagenesis, resulting in substantial loss of activity consistent with a catalytic role for these residues. Loss of activity of MDH in the presence of the reaction product morphinone was found to be due to the formation of a covalent adduct with Cys-80; alteration of Cys-80 to serine resulted in an enzyme with greatly enhanced stability.

Key words: biocatalysis, dehydrogenases, opiates, protein engineering.

INTRODUCTION

Pseudomonas putida M10 was isolated from opiate factory waste liquor on the basis of its apparent ability to grow with morphine alkaloids as sole carbon sources [1]. Although later studies failed to confirm this ability, M10 was found to transform morphine alkaloids to a number of potent semisynthetic opiate drugs. The first steps in the transformation of morphine and codeine are oxidation of the 6-hydroxyl group to a ketone by a NADP⁺-dependent morphine dehydrogenase (MDH) to give morphinone and codeinone (Figure 1) [1–3], and saturation of the 7,8-double bond by an NADH-dependent morphinone reductase to give hydromorphone and hydrocodone [4–6]. Oxidation of morphine by MDH was made the basis of a rapid and highly sensitive assay for opiates in urine [7,8], and the combined MDH-morphinone

reductase system has been applied to the biocatalytic production of the medically useful semisynthetic opiates hydromorphone and hydrocodone [9–11].

The structural gene encoding MDH, designated *morA*, was found to be located on a large natural plasmid, and was cloned and sequenced [3]. The sequence indicated that MDH belongs to the aldo-ketoreductase family of alcohol dehydrogenases [12], of which the best studied member is mammalian aldose reductase, which is implicated in complications of diabetes. Aldose reductase has been studied extensively and its crystal structure determined [13,14]. Mammalian aldehyde dehydrogenase and 3-hydroxysteroid dehydrogenase, yeast xylose reductase and *Corynebacterium* 2,5-diketo-D-gluconate reductase have also attracted considerable attention.

In an effort to improve the biotechnological processes based on MDH, we have undertaken a study of its catalytic properties. In the present study these properties are reported and compared with those of related enzymes. Further, we describe the production of a modified MDH with greatly improved stability under normal reaction conditions.

MATERIALS AND METHODS

Materials

MDH was purified from recombinant *Escherichia coli* as described previously [2]. Morphine alkaloids were kindly donated by Macfarlan Smith Ltd. (Edinburgh, U.K.). Other reagents were of analytical or higher grade.

Cofactor stereospecificity determination

NADPH deuterated in the pro-R position was prepared by the reaction of fully deuterated propan-2-ol (Sigma) with NADP⁺,

Figure 1 Reaction catalysed by morphine dehydrogenase

Abbreviation used: MDH, morphine dehydrogenase.

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catalysed by *Thermonaerobium brockii* secondary alcohol dehydrogenase (Sigma). The reaction mixture contained 20 mg/ml NADP⁺ and 2.5 units/ml alcohol dehydrogenase in 5 ml of 50 mM ammonium bicarbonate, pH 7.8/10% (v/v) deuterated propan-2-ol, and was incubated at 37 °C. When the absorbance at 340 nm had become constant (210 min), NADP²H was purified by anion-exchange chromatography [15] using a 10/10 Mono-Q column (Pharmacia) equilibrated with the same buffer. Pyridine nucleotides were eluted with a gradient of 50–500 mM ammonium bicarbonate, pH 7.8. The product was freeze-dried three times from ²H₂O and dissolved in ²H₂O for ¹H-NMR analysis. NMR was performed using a 400 MHz NMR spectrometer (JEOL Lambda400).

Reaction with codeinone was performed in a mixture containing 1.5 mM NADP²H, 0.7 mM codeinone and 0.3 mg/ml MDH in 50 mM ammonium carbonate buffer, pH 7.8, at 30 °C. When absorbance at 340 nm had become constant (19 min), alkaloids were extracted three times into chloroform and pooled. The solvent was removed by rotary evaporation. The residue was dissolved in a small volume of deuterated chloroform and analysed by ¹H-NMR as described above.

Steady-state kinetic analysis

MDH assays in the direction of hydroxy-alkaloid oxidation were performed in 50 mM glycine, pH 9.5. Assays in the direction of ketone reduction were performed in 50 mM phosphate buffer, pH 7.0. All assays were at 30 °C. Cofactor oxidation and reduction were followed at 340 nm using a diode array spectrophotometer (Hewlett Packard 8452A). Assays were performed in triplicate. Kinetic constants were calculated using the GraFit statistical package [16]. Alkaloids and cofactors were added at various concentrations as indicated in the Figure legends. One unit of enzyme activity was defined as that which oxidized 1 μmol of NADPH or reduced 1 μmol of NADP⁺ under specified conditions.

Site-directed mutagenesis

For site-directed mutagenesis of MDH, the Clontech Transformer kit was used according to the manufacturer's protocol. The oligonucleotides used were as follows: modification of Tyr-52 to Phe, 5'-CC GCC AGG TCG TTT AAC AAT GAG GC-3'; modification of Lys-76 to Met, 5'-C GTC ACC ACA ATG CTG TTC AAC TGC-3'; modification of Asp-47 to Asn, 5'-C CGT CTG ATC AAC ACC GCC AGG TCG-3'; modification of Lys-244 to Arg, 5'-GTT GCA ATT CCG CGG TCA GTC AAC CCC G-3'; modification of Lys-244 to Met, 5'-GTT GCA ATT CCG ATG TCA GTC AAC CCC G-3'; modification of Cys-80 to Ser, 5'-CTG TTC AAC TCC GAC TAT GGT TAC-3'; modification of Cys-140 to Ser, 5'-G ATC GGT GTA TCC AAC TTC CTG-3'.

Fluorescence titration

Tryptophan fluorescence of MDH in the presence of increasing cofactor concentrations was measured using a fluorescence spectrometer (Perkin-Elmer LS50B). Cuvettes contained 12–14 μg of enzyme in 4 ml of 50 mM phosphate buffer, pH 7.0. Measurements were at 30 °C. Excitation was at 280 nm with an excitation slit width of 2.5 nm and emission slit width of 5.0 nm. Change in fluorescence was measured over the range 300–500 nm, with scan speed 300 nm·min⁻¹, and measured at 340 nm. Cofactor was added in 1 μl portions from a 10 mM or 100 mM stock solution. The maximum total volume change was 2.6%. Gly-Trp dipeptide was used as a standard to correct for

dilution and inner-filter effects [17]. Data were fitted to the following equation by non-linear regression using the GraFit statistical package [16]:

$$\Delta F = \Delta F_{\max} [\text{NADP(H)}] / K_d + [\text{NADP(H)}]$$

where ΔF is the change in fluorescence.

Electrospray MS

Modification of MDH was performed using 3.75 μM enzyme in 1 ml of 50 mM glycine/NaOH buffer, pH 9.5, containing 3 mM morphine and 3 mM NADP⁺ at 30 °C. After the generation of morphinone *in situ*, the reaction mixture was maintained at 30 °C for 80 min. Samples were dialysed against water, concentrated to 24 μM and analysed using an electrospray mass spectrometer (VG Bio-Q). Electrospray MS was performed by the Department of Chemistry, Cambridge Centre for Molecular Recognition, University of Cambridge, U.K.

Enzyme inactivation studies

To study the inactivation of MDH by morphinone, it was necessary to generate morphinone *in situ*, since morphinone is difficult to synthesize chemically and is not commercially available. Reaction mixtures contained 1.5 μM MDH, 1 mM morphine and 1 mM NADP⁺ in 50 mM glycine, pH 9.5. Protection by cysteine was determined by adding cysteine at final concentrations of 0.5–4.0 mM. Aliquots were removed from the reaction mixture at regular time intervals and assayed for activity over a 5 s period to minimize further inactivation.

RESULTS AND DISCUSSION

Stereospecificity of hydride transfer

To determine the stereospecificity of hydride transfer in MDH, deuterated NADPH was prepared by reduction of NADP⁺ in the presence of fully deuterated propan-2-ol, using the secondary alcohol dehydrogenase of *T. brockii*, which is known to be pro-R specific [18]. Deuterated NADPH was purified by ion-exchange chromatography, and the presence of deuterium in the pro-R position was determined by ¹H-NMR [19].

The purified deuterated NADPH was used in the reduction of codeinone by MDH. Excess NADPH was used to drive codeinone reduction towards completion. Codeine was extracted and analysed by ¹H-NMR [20]. The peak at 4.18 p.p.m., due to the C-6 proton, was absent and the peak at 4.81 p.p.m., due to the C-5 proton, was unsplit, indicating that deuterium was present at position 6. We therefore concluded that MDH was specific for the pro-R hydrogen of NADPH. The same stereospecificity has been determined for aldose reductase [21] and 3α-hydroxysteroid dehydrogenase [22,23], and appears to be typical of this enzyme family.

Steady-state kinetic mechanism

To establish the steady-state kinetic mechanism of MDH, activity assays for both codeine oxidation and codeinone reduction were performed with various concentrations of substrate and cofactor. No substrate inhibition was observed at the concentrations used. For each cofactor concentration, apparent K_m and $V_{\max}$ were determined by non-linear regression using the GraFit statistical package [16].

For both oxidative and reductive assays, double-reciprocal plots showed a series of lines converging to the left of the axis (Figure 2), indicating a sequential mechanism (a mechanism in

Figure 2 Initial velocity studies of MDH

(A) Codeinone reduction. (●), 0.2 mM NADPH; (■), 0.5 mM NADPH; (△), 1 mM NADPH; (○), 2 mM NADPH; (□), 3 mM NADPH. (B) Codeine oxidation. (●), 0.2 mM NADP⁺; (■), 0.5 mM NADP⁺; (△), 1 mM NADP⁺; (○), 2 mM NADP⁺; (□), 3 mM NADP⁺. Assay mixtures contained 25 nM MDH. U, units. All points shown are the means of triplicate determinations. Curves were fitted independently for each cofactor concentration using the GraFit package [16].

which a ternary complex must be formed) [24]. Data were fitted to the appropriate equation by non-linear regression:

$$v = V_{\max} [A][B] / K_{ia} K_b + K_a [B] + K_b [A] + [A][B]$$

Table 1 Kinetic constants for reactions catalysed by MDH

Values are means $\pm$ S.E.M.

Reaction	K_{ia} (μ M)	K_a (μ M)	K_b (μ M)	$V_{\max}$ (units/mg of protein)	k_{cat} (s^{-1})
Morphine oxidation	539 $\pm$ 121	500 $\pm$ 39	482 $\pm$ 48	230 $\pm$ 7	123
Codeine oxidation	446 $\pm$ 129	366 $\pm$ 40	34 $\pm$ 4	168 $\pm$ 7	90
Codeinone reduction	119 $\pm$ 42	91 $\pm$ 9	22 $\pm$ 6	189 $\pm$ 14	101
MDH Cys-80 $\rightarrow$ Ser, codeine oxidation	192 $\pm$ 18	243 $\pm$ 43	24 $\pm$ 1	161 $\pm$ 2	85
MDH Cys-140 $\rightarrow$ Ser, codeine oxidation	257 $\pm$ 90	572 $\pm$ 40	49 $\pm$ 7	203 $\pm$ 8	108

Figure 3 Product inhibition studies of codeinone reduction

(A) Inhibition of MDH activity by NADP⁺ with NADPH as the varied substrate in the presence of 0.1 mM codeinone. (●), no NADP⁺; (△), 3 mM NADP⁺; (□), 6 mM NADP⁺; (○), 9 mM NADP⁺. (B) Inhibition of MDH activity by NADP⁺ with codeinone as varied substrate in the presence of 0.1 mM NADPH. Symbols as shown in (A). (C) Inhibition of MDH activity by codeine with NADPH as the varied substrate in the presence of 0.1 mM codeinone. (●), no codeine; (△), 3 mM codeine; (□), 6 mM codeine; (○), 9 mM codeine. (D) Inhibition of MDH activity by codeine with codeinone as the varied substrate in the presence of 0.1 mM NADPH. Symbols as for (C). Assay mixtures contained 25 nM MDH. U, units. All points are the means of triplicate determinations. Curves were fitted independently for each inhibitor concentration using the GraFit package [16].

where [A] is cofactor concentration, [B] is alkaloid concentration, and $V_{\max}$, K_{ia} , K_a and K_b are kinetic constants. These values are given in Table 1.

To determine the order of substrate binding, product inhibition studies were performed. Codeinone reduction with NADPH was assayed in the presence of various concentrations of NADP⁺ or codeine. For each NADP⁺ or codeine concentration, apparent $V_{\max}$ and K_m values were determined. The results are shown as double-reciprocal plots in Figure 3. NADP⁺ caused competitive inhibition with respect to NADPH and mixed (non-competitive) inhibition with respect to codeinone. Similarly, inhibition by codeine was competitive with respect to codeinone and mixed (non-competitive) with respect to NADPH. These results are

Figure 4 Product inhibition of codeine oxidation by codeinone

Codeine was the varied substrate in the presence of 0.1 mM NADP⁺: (●), no codeinone; (△), 10 μM codeinone; (□), 20 μM codeinone; (○), 50 μM codeinone. Assay mixtures contained 25 nM MDH. U, units. All points are the means of triplicate determinations. Curves were fitted independently for each inhibitor concentration using the GraFit package [16].

consistent with either a random-binding mechanism, in which either alkaloid or cofactor can bind first, or a Theorell–Chance mechanism, in which binding order is obligatory but the ternary complex does not accumulate [24].

A sequential ordered mechanism with cofactor binding first has been demonstrated for aldose reductase [25,26], aldehyde reductase [27] and 3 α -hydroxysteroid dehydrogenase [23], although, in the cases of aldose reductase and hydroxysteroid dehydrogenase, alternative evidence has suggested a random binding mechanism and/or formation of enzyme-substrate complexes in the absence of cofactor under some circumstances. In the case of MDH, inhibition by codeine of codeinone reduction at pH 7 is competitive with respect to codeinone, suggesting that codeinone is able to prevent codeine from binding to the enzyme–NADP⁺ complex. This implies a random binding mechanism. An alternative possibility is that the reaction displays Theorell–Chance kinetics, with reaction and alkaloid product dissociation occurring much more rapidly than cofactor release. This is plausible, since cofactor release in aldose reductase has been shown to require an isomerization of the enzyme–NADP⁺ complex [25,26] which is rate-limiting in the direction of aldose reduction.

To clarify this problem, inhibition of codeine oxidation in the presence of codeinone was determined (Figure 4). Codeinone was found to cause mixed (non-competitive) inhibition with respect to codeine. This is consistent with a sequential ordered mechanism with cofactor binding first, and inconsistent with a random binding mechanism. It therefore seems most likely that, at pH 7.0, a Theorell–Chance mechanism operates and, at pH 9.5, the relative rates of the reaction steps are altered so that a ternary complex accumulates to a significant degree and the reaction displays a standard sequential ordered mechanism.

Catalytically important residues

Studies of aldose reductase have shown that residues Tyr-48, Lys-77 and Asp-43 are important for catalysis [13,28], and these residues are conserved in related enzymes, including MDH [12]. Tyr-48 is believed to act as the proton donor for the reductive

Table 2 Dissociation constants for cofactor binding to MDH

The values shown are means $\pm$ S.E.M.

Enzyme	K_d NADP ⁺ (μ M)	K_d NADPH (μ M)	K_d NADP ⁺ / K_d NADPH
Wild-type MDH	250 $\pm$ 58	67 $\pm$ 8	4.6
MDH Tyr-52 $\rightarrow$ Phe	519 $\pm$ 60	146 $\pm$ 15	3.5
MDH Lys-76 $\rightarrow$ Met	153 $\pm$ 20	308 $\pm$ 50	0.5
MDH Asp-47 $\rightarrow$ Asn	187 $\pm$ 31	45 $\pm$ 4	4.2

reaction, and interaction with the side-chain of Lys-77 is believed to decrease the pK_a of the tyrosine side-chain to facilitate this reaction [28]. Asp-43 also participates in a hydrogen-bonding network with Tyr-48 and Lys-77 [29]. In aldose reductase, mutant Tyr-48 $\rightarrow$ Phe is catalytically inactive and Lys-77 $\rightarrow$ Met has much lower activity [28,29]. Aldose reductase Asp-43 $\rightarrow$ Asn has somewhat lower activity and less affinity for cofactors [29].

The equivalent residues in MDH are Tyr-52, Lys-76 and Asp-47. To investigate the importance of these residues for MDH activity, site-directed mutagenesis was used to generate mutant forms of MDH with the following alterations: Tyr-52 $\rightarrow$ Phe, Lys-76 $\rightarrow$ Met and Asp-47 $\rightarrow$ Asn. The mutant enzymes were purified to electrophoretic homogeneity in the same way as wild-type MDH. The identity of the purified proteins was confirmed by N-terminal sequencing.

No activity was detected in MDH Tyr-52 $\rightarrow$ Phe. Slight morphine oxidation activity was detected in MDH Lys-76 $\rightarrow$ Met and Asp-47 $\rightarrow$ Asn. Kinetic parameters could not be determined due to the extremely low activity and the poor solubility of morphine. With 3 mM morphine and 3 mM NADP⁺, the measured NADP⁺ reduction rates with MDH Lys-76 $\rightarrow$ Met and Asp-47 $\rightarrow$ Asn were 0.03% and 0.96% of wild-type MDH respectively. These results are consistent with a catalytically important role for these residues.

To confirm that loss of activity was not due to major structural alterations, binding of cofactors to the mutant enzymes was investigated by fluorescence titration. Dissociation constants for the binding of cofactors to wild-type and mutant enzymes are shown in Table 2. During titration with NADPH, loss of fluorescence at 340 nm coincided with an increase in the NADPH emission peak at 460 nm (results not shown), indicating that the observed loss of fluorescence was at least partially due to energy transfer to NADPH. However, loss of fluorescence was also observed during titration with NADP⁺, which is essentially non-fluorescent, suggesting that loss of fluorescence was due to a conformational change.

Dissociation constants for the wild-type enzyme are consistent with those estimated from steady-state kinetic analysis on the assumption of an ordered sequential mechanism, in which K_{ia} is equal to the dissociation constant for the first substrate to bind. Dissociation constants for the mutant enzymes were similar to those for wild-type MDH, indicating that the mutations did not greatly interfere with cofactor binding. This argues against the observed loss of activity being due to major structural alterations and suggests that disruption of catalytic activity is a more likely explanation.

As in aldose reductase [29], MDH Lys-77 $\rightarrow$ Met showed lower affinity for NADPH and increased affinity for NADP⁺, and MDH Tyr-52 $\rightarrow$ Phe showed decreased affinity for NADP⁺. In contrast to aldose reductase, MDH Tyr-52 $\rightarrow$ Phe showed a considerably decreased affinity for NADPH, and the mutation

Figure 5 Inactivation of MDH during morphine oxidation

(▲), no cysteine; (■), 0.5 mM cysteine; (●), 1 mM cysteine; (△), 2 mM cysteine; (□), 4 mM cysteine; (○), no morphine. Activities are relative to the activity of MDH at zero time, which is taken as 100%.

Asp-43 → Asn, which, in aldose reductase, causes a 5- to 6-fold lowering of affinity for both cofactors, appeared, in MDH, to increase slightly the affinity for both NADPH and NADP⁺. This implies subtle differences in the nature of cofactor binding between the two enzymes.

The crystal structure of aldose reductase showed that residue Lys-262 is involved in binding the 2'-phosphate of NADP(H) [13]. To investigate the role of the corresponding residue in MDH (Lys-244) the mutants Lys-244 → Met and Lys-244 → Arg were generated and purified. Both of these mutated enzymes showed lower morphine oxidation activity compared with wild-type MDH. Again, kinetic parameters could not be determined, since the apparent K_m for morphine exceeded the solubility of morphine in the reaction buffer. With 1 mM morphine and 1 mM NADP⁺, the Lys-244 → Met mutation had 0.2% and Lys-244 → Arg 11.7% of the activity of wild-type MDH; with 3 mM morphine and 3 mM NADP⁺, the corresponding values were 1.6% and 19.8%. Thus MDH Lys-244 → Met had a markedly lower activity, as was reported previously for aldose reductase and aldehyde reductase [30]. MDH Lys-244 → Arg, in which the positive charge of the sidechain is conserved, showed considerably greater activity. This is consistent with a role for the positive charge of Lys-244 in binding of NADP(H). Fluorescence titrations were performed to estimate the affinity of these mutated enzymes for cofactors. In contrast to the mutants Lys-77 → Met, Tyr-52 → Phe and Asp-43 → Asn described above, mutants Lys-244 → Arg and Lys-244 → Met showed much lower cofactor binding, and accurate dissociation constants could not be obtained.

The results of these experiments suggest that MDH is generally similar to mammalian aldose reductase in terms of cofactor binding and catalysis.

Inactivation of MDH by morphinone

MDH rapidly lost activity during the morphine oxidation reaction. Loss of activity could be diminished by the presence of

Figure 6 Inactivation of wild-type MDH, MDH Cys-80 → Ser and MDH Cys-140 → Ser during morphine oxidation

(△), wild-type; (○), Cys-80 → Ser; (□), Cys-140 → Ser. All activities are relative to wild-type MDH at zero time, which is taken as 100%.

free cysteine (Figure 5), suggesting that the product morphinone, a reactive α/β -unsaturated ketone [31], which is known to react readily with thiol groups by Michael addition [32,33], was interacting with a cysteine sidechain. MDH was analysed by electrospray MS before and after being used in morphine oxidation reactions. The predicted M_r of wild-type MDH, based on sequence data, is 31993. The M_r of freshly purified wild-type MDH was determined as 32003 ± 10 . After the catalysis of morphine oxidation, the M_r was 32279 ± 5 , an increase of 276 ± 15 over the measured value or 286 ± 5 over the predicted value. This is consistent with binding of a single molecule of morphinone (M_r 283). The mass spectroscopy data indicated that essentially all of the enzyme had been modified over the 80 min incubation period. Protection by free cysteine suggested that morphinone is released from the active site before reaction with the enzyme.

A similar phenomenon has been described in the related enzyme, 3- α -hydroxysteroid dehydrogenase [34]. The unsaturated alcohols 1-(4'-nitrophenyl)prop-2-en-1-ol and 1-(4'-nitrophenyl)propyn-1-ol have been tested as specific enzyme inhibitors. The dehydrogenase activity converts these alcohols into α/β -unsaturated ketones which covalently modify the enzyme at the cofactor binding site. The enzyme is protected in the presence of 2-mercaptoethanol.

MDH contains two cysteine residues. By comparison with the aldose reductase structure, Cys-80 is likely to be on the enzyme surface and Cys-140 is predicted to lie within the substrate binding pocket. The two cysteine residues were altered to serine by site-directed mutagenesis, and the mutated enzymes, MDH Cys-80 → Ser and Cys-140 → Ser, were purified to homogeneity. Both mutants showed activity at similar levels to wild-type MDH. Kinetic parameters were measured as for wild-type MDH (Table 1). The M_r of both mutants was determined by electrospray MS before and after reaction with morphine. The predicted M_r in both cases is 31976.5. Following the morphine oxidation reaction, the M_r of MDH Cys-80 → Ser was 31976 ± 7 and that

of MDH Cys-140 → Ser was 32263 ± 6 , an increase of 286 ± 6 . This suggests that Cys-80 is the normal site of attack by morphinone.

In reaction mixtures containing 3 mM morphine and 3 mM NADP⁺, MDH Cys-80 → Ser lost activity much less rapidly than MDH Cys-140 → Ser or wild-type MDH (Figure 6). In other respects, MDH Cys-80 → Ser shows similar kinetic properties to wild-type MDH (Table 1). Thus the change of cysteine to serine significantly improved stability. We therefore anticipate that this enzyme mutation will offer significant advantages over wild-type MDH for commercial use.

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