Substrate and Temperature Dependence of DNA Photolyase Repair Activity Examined with Ultrafast Spectroscopy

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Abstract: The kinetics and energetics of the electron transfer reactions occurring in the enzyme·substrate complex of DNA photolyase have been studied by transient absorption spectroscopy with picosecond time resolution in the temperature range 275−90 K. The results can be summarized as follows: (i) The lifetime of $^1(FADH^-)^*$ depends not only on the presence of the substrate but also on its nature, e.g. the electron transfer from $^1(FADH^-)^*$ to thymine dimers T<>T and to thymine-uracil dimers T<>U is slower by a factor of 2 to 3 compared to that of U<>U and U<>T. This feature is attributed to the enlarged electronic coupling between $^1(FADH^-)^*$ and the respective dimers in the absence of the methyl group in the C5 position of the 5′-uracil. (ii) The temperature dependence of the quantum yield was studied, and from this the activation energy for the overall repair process has been estimated to be $E_A = 0.45 \pm 0.1$ eV.

Introduction

Solar ultraviolet light (290−400 nm) is known to have mutagenic, carcinogenic, and lethal effects.[1] The main targets of UVA and UVB radiation are the pyrimidine DNA bases, with cyclobutane type pyrimidine dimers (Pyr<>Pyr) and the 6−4 photoadduct (pyrimidine (6−4) pyrimidones) the most common products.[2] In the case of the pyrimidine dimers, one enzyme system in particular has been the subject of intense study, namely the DNA photolyases.[3] These enzymes are of especial interest as they are one of the few enzymes to utilize the energy of a photon (λ = 300−500 nm). Their mode of action involves the cleavage of the cyclobutane ring of the pyrimidine dimer directly without excision of the damaged base.

The general features of the photochemical reactions involved in DNA repair are now well-known.[4] However, there are several aspects of the reaction mechanism that remain to be clarified. In particular, in the early days of investigations on photolyase, a pronounced temperature dependence of the enzyme activity was inferred from studies on cell free enzyme extracts.[5] More recently, the structure of the substrate has been shown to have a significant influence on the overall efficiency of dimer splitting.[6] These observations prompted us to study in more detail the kinetics of the primary electron transfer reaction of *E. coli* DNA photolyase.

Experimental Section

Enzyme Preparation. *E. coli* photolyase was prepared as described previously.[7] The folate depleted apoenzyme was prepared by treating the enzyme with a 3−5 stoichiometric excess $NaBH_4$ from a 1 M stock solution with 50 mM $Na_2B_4O_7$, and dialyzing sufficiently in enzyme storage buffer (50 mM Tris−HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM Dithiothreitol and 50 v/v glycerol) until the absorption peak at 380 nm did not decrease any more.

Substrate Preparation. The cis-syn cyclobutane pyrimidine dimers were prepared by acetone-sensitized irradiation with a UVB lamp (8 W, 302 nm) (UVP). The TT, TU, dUT, and d(UU) dinucleotides were synthesized on a solid-phase DNA synthesizer (Applied Biosystems) and purified on a reverse-phase C-18 column (Dynamax-300A, 4.6 × 250 mm) (RAININ) by HPLC. The purified starting materials were dissolved in 10% aqueous acetone at a concentration of 0.5 mM and degased by purging N_2 gas for 20 min, and then put in a petri dish (100 × 15 mm) that was sealed in a ziplock bag full of N_2 gas. The dinucleotide was irradiated on ice under a UVB lamp. After the disappearance of the distinctive 260-nm peak, which was monitored by UV absorption, the reaction was stopped and the solvent was evaporated. The major photoproduct was isolated from the reaction mixture on reverse phase HPLC with a 75 mM potassium phosphate buffer (pH 6.8) and then desalted by washing with water on the same C-18 column. The pump flow rate was 1.0 mL/min, and the detector wavelength was 230 nm.

The photoproducts were characterized by both ^{1}H NMR spectroscopy and photoreactivation with *E. coli* photolyase. In the reaction buffer (50 mM Tris−HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM Dithiothreitol), the 260-nm absorption was recovered after the photoreactivation of the pyrimidine dimer with excess photolyase under Sylvania black light (λ_{max} = 366 nm). This confirmed the cis-syn stereochemistry of the substrate. The concentrations of the dimer substrates were also calculated on the basis of the recovered 260-nm absorption. ^{1}H NMR spectra of the cis-syn pyrimidine dimers were taken on a Bruker 500-MHz spectrometer (D_2O, referenced to DHO at

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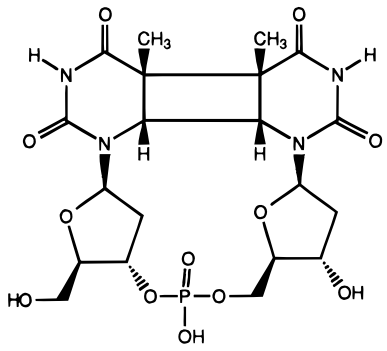
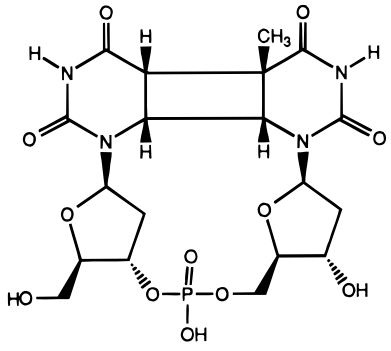
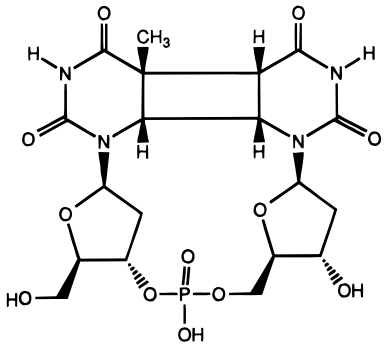
Chart 1. Cis-Syn Cyclobutane Pyrimidine Dimers Used in This Study



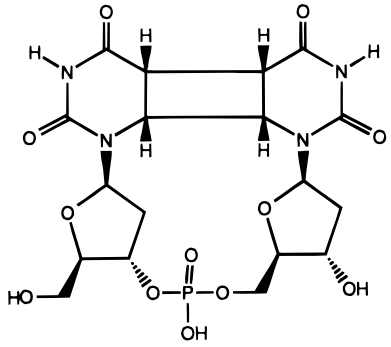
T<>T



U<>T



T<>U



U<>U

4.8 ppm), ^{1}H NMR of T<>T and T<>U were identical with the reported[8,9] ^{1}H NMR of U<>T: δ 6.04 (dd, J = 7.6 Hz, 1H, H1), 5.65 (dd, J = 9.0, 5.2 Hz, 1H, H1), 4.68 (m, 1H), 4.46−4.41 (m, 2H), 4.28 (m, 1H), 4.21 (m, 1H), 4.12 (m, 1H), 3.97 (m, 2H), 3.77−3.68 (m, 2H), 3.51 (d, J = 7.7 Hz, 1H), 2.71 (m, 1H), 2.37−2.32 (m, 2H), 2.22 (m, 1H), 1.66 (s, 3H, TCH_3). ^{1}H NMR of U<>U: δ 6.00 (dd, J = 6.1 Hz, 1H, H1), 5.62 (dd, J = 9.5, 4.7 Hz, 1H, H1), 4.73−4.70 (m, 2H), 4.55 (m, 1H), 4.32 (m, 1H), 4.24 (m, 1H), 4.13 (m, 1H), 4.04−3.96 (m, 3H), 3.88 (m, 1H), 3.75−3.71 (m, 2H), 2.60 (m, 1H), 2.42−2.33 (m, 2H), 2.17 (m, 1H).

Picosecond Time Resolved Absorption Spectroscopy. The reduced cofactor of DNA photolyase ($FADH^-$) was excited with the third harmonic of a Nd:YAG laser (355 nm) with an intensity of about 50 $\mu J/mm^2$. An intensity of more than 100 $\mu J/mm^2$ caused oxidation of $FADH^-$ to $FADH^0$. The repetition rate was about 2.5 Hz. There was no detectable difference to measurements with a 0.5 Hz repetition rate.

The difference absorbance was probed with a dye-laser, which was also pumped by the third harmonic of the Nd:YAG. By using the dye PBBO in 7:3 toluol:ethanol the laser was tunable in the region from 390 to 420 nm. The probe beam was optically delayed against the excitation by up to 24 ns. The pulse length of both the excitation and probe beams was 100 ps.

Due to the high quantum yield of dimer repair at room temperature, repetitive excitation of a given sample volume containing enzyme and substrate leads to the complete repair of the substrate at the excited spot. Therefore, to study the dynamics of electron transfer from $FADH^-$ to the dimer species the sample was moved after 25 shots. Under such conditions the difference absorption was identical with the one measured when moving the sample after each shot. All together 100 measurements on four different sample volumes were used to create one data point.

Results

Decay of $FADH^-$* and $FADH^-$*/Substrate at 275 K. We used four different types of *cis-syn* cyclobutane pyrimidine dimer substrates (Chart 1) to investigate the kinetics and energetics of electron transfer reactions in the photolyase−substrate complex. In the absence of substrate the excited singlet state of $FADH^-$ decays with the time constant of 1.8 ns. This decay is accelerated by more than a factor of 10 in the presence of pyrimidine dimers as long as sufficient concentration ensures that each enzyme had a substrate bound. In fact, the increased rate of decay results in an apparent diminished amplitude of the difference absorbance after 100 ps, due to the limited time resolution of the instrument (100 ps).

The effect of substrate was investigated in more detail by comparing the decay of excited $FADH^-$ in the presence of pyrimidine dimers both before and after dimer splitting. Hence the latter experiment is equivalent to the absence of dimers, but has the advantage that variations in sample preparation are avoided. Figure 1 shows the decay of $FADH^-$* in the presence of T<>T in the same sample both before and after dimer repair. In the former case, the sample was moved and the observed decay can be fitted to a biexponential function. The short time component is about 110(+70,−50) ps. As free enzyme is assumed to be responsible for the long time component, its lifetime was fixed to 1.8 ns in the fitting procedure. Subsequently, the repetitive excitation at the same spot repairs the substrate and leads to the recovery of the amplitude of the FADH absorption and the characteristic decay pattern observed for the free enzyme.

In the presence of U<>U (Figure 2) the apparent amplitude is decreased further relative to T<>T, due to the even faster decay of $FADH^-$*. Although the decay rate is too fast to accurately determine its time constant, an approximate value of 50 ps can be estimated. Similarly, an estimated decay time of 100 ps was derived in the presence of T<>T.

Decay of $FADH^-$* and $FADH^-$*/Substrate at 90 K. At 90 K the decay in the presence of substrate is slowed down by

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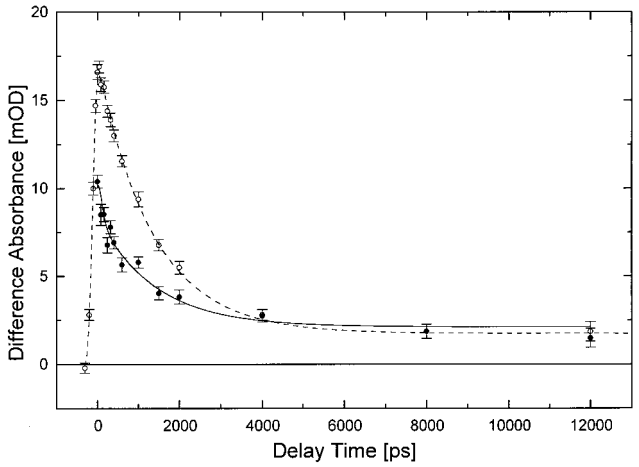

Figure 1. Time course of difference absorbance of $FADH^-$ probed at 410 nm and 275 K with (●) and without (○) T<>T bound to the enzyme. Enzyme: 0.18 mM. T<>T: 0.9 mM, 50% glycerol. The curves are biexponential fits.

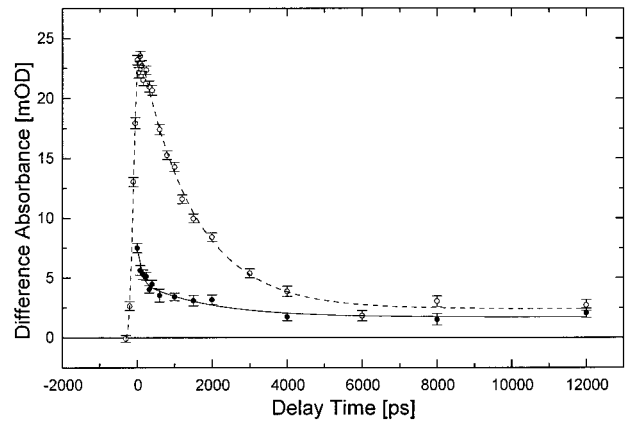

Figure 2. Time course of difference absorbance of $FADH^-$ probed at 410 nm and 275 K with (●) and without (○) U<>U bound to the enzyme. Enzyme: 0.27 mM. U<>U: 2 mM, 50% glycerol. The curves are biexponential fits.

a factor of between 3 and 6 (depending on the substrate) compared to the rate at 275 K. Nevertheless, it is still faster than in the absence of dimers by more than a factor of 10, the excited state lifetime of $FADH^{-}$* being 7.5 ns at 90 K. In the case of T<>T a biexponential fit reveals a short component of 310 ± 40 ps at 170 K and of 360 ± 60 ps at 90 K. In the case of U<>U the decay is also slower (150 ps) at lower temperature, although the small amplitude of the signal still prevented a precise determination even at this low temperature (Figure 3).

The decay rate of $FADH^-$ was also determined in the presence of the heterodimers T<>U and U<>T. Of particular interest (Figure 3) is that the experimental results for T<>U are similar to that for T<>T, with time constants of 80 ± 30 ps at 275 K, 460 ± 120 ps at 170 K, and 510 ± 90 ps at 90 K, whereas U<>T resembles U<>U with a very fast component (50 ps) at 275 K and 130 ps at 90 K (Table 1).

The Temperature Dependence of the Photorepair Process. As noted earlier, at room temperature, repetitive excitation at the same spot leads to dimer repair as evidenced by the recovery in the amplitude (at 100 ps) of the signal due to $FADH^{-}$*. Hence the extent of recovery of this signal as a function of the number of laser excitations at the same spot was employed as a measure of the photorepair efficiency. As can be seen from Figure 4 with U<>T as a substrate, while rapid dimer repair occurs at room temperature, at lower temperatures the increase in the amplitude of $FADH^{-}$* is less pronounced and below 200 K it could not be detected at all. Control experiments carried out at 180 K on completely repaired dimer demonstrated that the observed changes in signal amplitude during repair as a function of temperature were not due to changes in the intrinsic signal amplitude of $FADH^-$ in the absence of substrate.

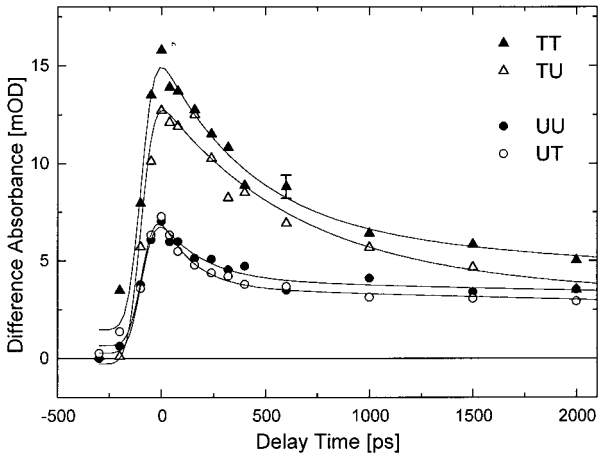

Figure 3. Time course of the transient difference absorbance of $FADH^-$ at 410 nm and 90 K with T<>T, T<>U, U<>U, and U<>T bound to the enzyme. The curves are biexponential fits.

Table 1. Measurement of the Transient Difference Absorbance of $FADH^-$ in the Presence of Various Substrates: Results of Biexponential Fits for the Short Time Constant

substrate	temp, K	short time constant from fit, ps	short time constant from amplitude, ps	long time constant (fixed in fit), ns
U<>U	275	<100	≈50	1.8
	90	170 ± 80		7.5
U<>T	275	<100		1.8
	90	130 ± 30		7.5
T<>T	275	110(+70,−50)	≈100	1.8
	170	310 ± 40		7.5
	90	350 ± 60		7.5
T<>U	275	80(+30,−20)		1.8
	170	460 ± 120		7.5
	90	510 ± 90		7.5

[a] The long time constant was assumed to be the decay time of the free $FADH^{-}$* and was fixed during the fit. Two estimates of the short time constant are given, based upon fitting the decay curve and the decrease of the amplitude.

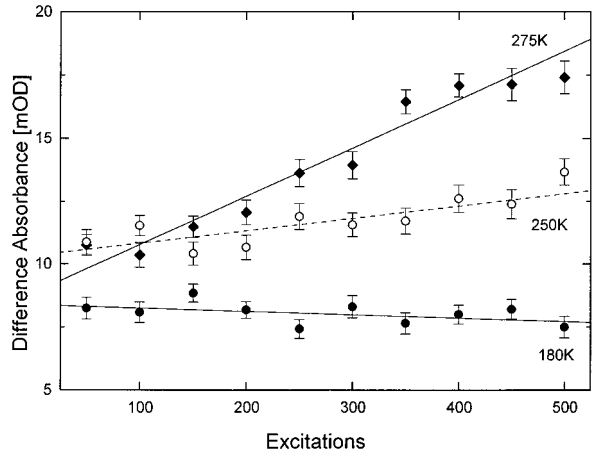

Figure 4. Maximum of difference absorbance (after 100 ps) depending on the number of excitations at different temperatures. One excitation gives 25 μJ of UV light on the sample. Omitted for clarity are the curves for 90, 240, and 260 K. Enzyme: 0.27 mM, Substrate U<>T: 1.35 mM, 50% glycerol. The curves are linear fits. At 275 K it is considered that the increase saturates.

Discussion

The Difference between Electron Transfer Rates to T<>T, T<>U and U<>U, U<>T. The results have shown that the forward electron transfer from $FADH^{-}$* to U<>U and U<>T is faster than that to T<>T and T<>U. In the nonadiabatic electron transfer theory the rate k_{FET} is determined by the electronic coupling V between donor and acceptor and the Franck−Condon factor (FC):[10]

$$k_{FET} = \frac{2\pi}{\hbar}|V|^2 \cdot FC \qquad (1)$$

In principle, the variation in rate constant of electron transfer to four different substrates could be due to differences in either *V* or the FC factor. In the first case, changes in the enzyme/substrate docking geometry are involved, while in the second case differences in ΔG and/or λ would be predominant. Although small differences in redox potential for U<>U and T<>T are expected (0.1 V),[11] the redox potentials of U<>T and T<>U are expected to be almost exactly the same. Hence assuming that ΔG and λ have the same value for each of the different substrates, the electronic coupling *V* would be responsible for the difference of k_{FET}, and would reflect a slightly larger distance (*R*) between $FADH^{-}$* and the substrate according to

$$V = V_0 e^{-\alpha R} \qquad (2)$$

The value of α would be expected to be about 0.7 $Å^{-1}$ for proteins.[12] It is suggested therefore that T<>U docks with photolyase in a less favorable orientation than does U<>T. Such a variation of the $FADH^{-}$ to substrate distance would be consistent with steric hindrance of the methyl group when in the 5' rather than the 3' base and explain the faster rates for U<>U and U<>T. This suggests that after being abstracted from $FADH^{-}$*, the electron may be located on the 5' rather than the 3' base. A similar explanation was previously proposed based on data from model studies of pyrimidine dimer splitting with N^{α}-acetyltryptophan as an electron-donating sensitizer.[13]

It is known that under steady-state conditions T<>T is repaired faster than T<>C, which in turn is repaired faster than C<>C.[3c] It appears that both K_m and k_{cat} effects contribute to these different rates of repair.[6] However, similar steady-state measurements for uracil-containing dimers have not been conducted. Equilibrium binding measurements have yielded binding constants for T<>T, T<>U, and U<>U within a factor of 2 of one another;[6] there are no reported measurements for the U<>T photodimer. Hence further work is required to evaluate the contribution of the various forward electron transfer rates observed in this study to the steady-state rates of repair of various dimers.

Estimate of the Activation Energy of Repair. In the present work no dimer splitting was observed below 200 K. This phenomenon also has been reported in the context of low-temperature ESR measurements.[14] On the other hand, it should be noted that pyrimidine dimers in LiCl glass still split at 77 K upon one-electron[15] reduction in pulse radiolysis experiments.

The interpretation of the temperature dependence of the complex dimer repair is difficult since quite different processes and therefore rates have to be considered, as e.g. k_{FET}, the rates of dimer splitting and back-electron transfer, k_{SPL}, and k_{BET}, respectively, as well as the desorption kinetics of pyrimidine monomers after repair.

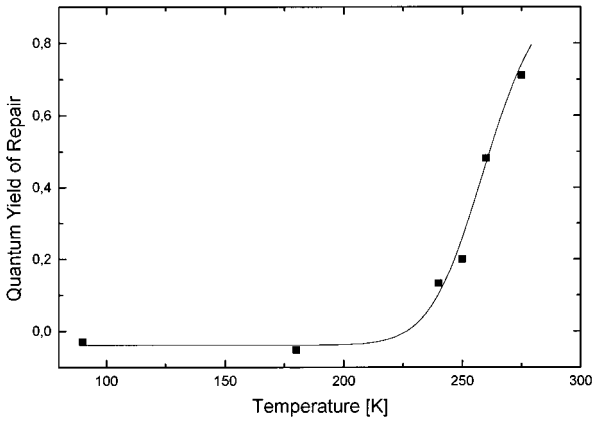

Figure 5. Optimum yield of repair depending on temperature with U<>T bound to the enzyme. The results of Figure 5 and measurements of the yield at 285 K[6] are used to calculate the yield (eq 4).

The only direct information relates to k_{FET}, which has been shown in this paper to decrease by a factor of 3−6 upon cooling from 275 to 90 K. Since the lifetime of $FADH^{-}$* without substrate also increases by a factor of about 4−5, forward electron transfer cannot be responsible for the decrease of the quantum yield of repair with decreasing temperature.

If back-electron transfer takes place in the inverted region as postulated in ref 3b, only a negligible temperature dependence is expected. Then, in the simplest approach to the temperature dependence of the quantum yield of repair, $\Phi(T)$ would mirror predominantly the temperature dependence of the splitting rate:

$$\Phi(T) = \frac{k_{Sp}(T)}{k_{BET} + k_{Sp}(T)} \qquad (3)$$

With $k_{Sp}(T) = k^0_{Sp} e^{-E_A/kT}$ we obtain

$$\Phi(T) = \frac{1}{(k_{BET}/k^0_{Sp})e^{E_A/kT} + 1} \qquad (4)$$

The relative efficiencies of repair obtained in this work can be converted to absolute quantum yields of repair by calibration to $\Phi(285K) = 0.8$ for U<>T.[6] The quantum yields fitted to eq 4 are shown in Figure 5, yielding an activation energy $E_A = 0.45 \pm 0.1$ eV for U<>T. For U<>U the same treatment yields $E_A = 0.45 \pm 0.2$ eV (data not shown).

The activation energy is the energetic barrier for the ground state radical anion of the pyrimidine dimer substrate to split into monomers. Under the oversimplified condition, the temperature dependence of k_{FET} and k_{BEF} is negligible. Although the splitting of the radical anion is 9 kcal/mol more exergonic than that of the neutral dimer to monomers,[11] 0.45 eV (=10.7 kcal/mol) is still needed to overcome the splitting energy barrier, which is easily compensated at ambient temperature. Therefore the whole photoreversion process has been regarded as a "photon powered" reaction.[3b] This simplification is somewhat misleading when the reaction is performed at low temperature, for example below 200 K. The temperature dependence of the quantum yield or dimer splitting efficiency is consistent with the notion that the actual bond breaking is the rate determining step of this reaction. This step needs energy supplied from an external source; otherwise, the reaction is trapped in the

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equilibrium between the forward and back-electron transfer process without leading to dimer splitting, as we observed at temperature below 200 K. Hence the lack of repair at low temperatures might indeed reflect a large activation energy E_A of splitting. At low temperatures, this high activation energy hinders the competition of splitting with back-electron transfer.

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