Quantum catalysis in B₁₂-dependent methylmalonyl-CoA mutase: experimental and computational insights

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B₁₂-dependent methylmalonyl-CoA mutase catalyses the interchange of a hydrogen atom and the carbonyl-CoA group on adjacent carbons of methylmalonyl-CoA to give the rearranged product, succinyl-CoA. The first step in this reaction involves the transient generation of cofactor radicals by homolytic rupture of the cobalt–carbon bond to generate the deoxyadenosyl radical and cob(II)alamin. This step exhibits a curious sensitivity to isotopic substitution in the substrate, methylmalonyl-CoA, which has been interpreted as evidence for kinetic coupling. The magnitude of the isotopic discrimination is large and a deuterium isotope effect ranging from 35.6 at 20 °C to 49.9 at 5 °C has been recorded. Arrhenius analysis of the temperature dependence of this isotope effect provides evidence for quantum tunnelling in this hydrogen transfer step. The mechanistic complexity of the observed rate constant for cobalt–carbon bond homolysis together with the spectroscopically silent nature of many of the component steps limits the insights that can be derived by experimental approaches alone. Computational studies using a newly developed geometry optimization scheme that allows determination of the transition state in the full quantum mechanical/molecular mechanical coordinate space have yielded novel insights into the strategy deployed for labilizing the cobalt–carbon bond and poisoning the resulting deoxyadenosyl radical for subsequent hydrogen atom abstraction.

Keywords: cobalamin; B₁₂; tunnelling; methylmalonyl-CoA mutase

1. INTRODUCTION

Coenzyme B₁₂ or 5'-deoxyadenosylcobalamin (AdoCbl)-dependent enzymes catalyse the 1,2 rearrangement of diverse substrates which involves the interchange of a hydrogen atom and a variable group (–OH, –NH₂ or a carbon-containing fragment) on vicinal carbons (figure 1; Banerjee & Ragsdale 2003). During the reaction, the hydrogen atom journeys intermolecularly from the substrate to the deoxyadenosyl moiety of the cofactor before returning to the adjacent carbon on the substrate to give the isomerized product. In contrast, the variable group migrates intramolecularly. Nature has met the challenge of these chemically difficult reactions by deployment of radicals; hence the use of coenzyme B₁₂, which serves as a latent radical reservoir (Halpern 1985).

Coenzyme B₁₂ belongs to the family of tetrapyrrolic-derived organometallic cofactors and has a cobalt atom that is coordinated equatorially to the nitrogens of the macrocycle. The reactivity of this cofactor resides in the metalloalkyl cobalt–carbon bond, which is estimated to have a bond dissociation energy of *ca* 31.5 kcal mol^{–1} (Finke & Hay 1984). Homolytic rupture of this bond generates cob(II)alamin and the deoxyadenosyl radical (figure 1, *step i*). The latter, a primary reactive radical,

initiates the isomerization reaction by the abstraction of a hydrogen atom from the substrate to yield a substrate-centred radical (*step ii*). This represents the first of two hydrogen atom transfer steps and occurs between the substrate and the cofactor. The substrate radical then undergoes rearrangement, in a step in which the variable group migrates intramolecularly between vicinal carbons to yield a product-centred radical (*step iii*). The latter then reabstracts a hydrogen atom from deoxyadenosine in the second hydrogen transfer step (*step iv*). Recombination of the cofactor-based radicals completes the turnover cycle (*step v*).

The specific rearrangement catalysed by methylmalonyl-CoA mutase is the reversible interconversion of methylmalonyl-CoA and succinyl-CoA (Banerjee 2003). This is the only known AdoCbl-dependent reaction found in mammals, where it serves to funnel catabolites of branched-chain amino acids, odd-chain fatty acids and cholesterol to succinyl-CoA, which can be used in central carbon metabolism. This enzyme is also prevalent among microbes, where it is important in the reverse metabolic direction, linking the production of propionate to succinate (Allen *et al.* 1964).

A prediction of the mechanism shown in figure 1 is that it involves biradical intermediates in which the metal-based radical, cob(II)alamin, plays a sedentary role whereas the reactive organic radical propagates from the cofactor to the carbons in the substrate and product radicals, respectively. This mechanistic proposal is supported by the detection by electron

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Figure 1. Postulated reaction mechanism of methylmalonyl-CoA mutase. MCoA and SCoA refer to methylmalonyl-CoA mutase and succinyl-CoA, respectively.

paramagnetic resonance (EPR) spectroscopy of a radical pair intermediate under steady-state turnover conditions (Keep *et al.* 1993; Zhao *et al.* 1994; Padmakumar & Banerjee 1995). Based on the selective isotopic labelling of substrates, the EPR spectrum is assigned to a spin-coupled radical pair between cob(II)alamin and the succinyl-radical (Mansoorabadi *et al.* 2005).

2. KINETICS AND THERMODYNAMICS OF THE HYDROGEN TRANSFER STEPS

The mechanism as described in figure 1, minimally involves two hydrogen atom transfer steps (*ii* and *iv* in figure 1). The overall deuterium isotope effect ($^D V$) for the methylmalonyl-CoA mutase-catalysed reaction measured under non-competitive conditions has been reported to be 5–6 in the forward direction (Michenfelder *et al.* 1987; Chowdhury *et al.* 2001) and 3.4 in the reverse direction at 30 °C (Thomä *et al.* 1998). A steady-state tritium kinetic isotope effect (KIE; $k_{\text{H}}/k_{\text{T}}$) of 3.2 at 30 °C has been reported in the reverse direction (Meier *et al.* 1996). Under steady-state turnover conditions, the intrinsic isotope effect is suppressed, presumably by product release which is believed to be rate limiting. A large deuterium isotope effect has been measured under pre-steady-state conditions when the holoenzyme is rapidly mixed with substrate and formation of the homolysis product, cob(II)alamin, is monitored (Padmakumar *et al.* 1997). Under these conditions, the isotope effect is 35.6 at 20 °C and 49.9 at 5 °C (Chowdhury & Banerjee 2000a,b). These measurements could not be

performed with sufficient accuracy at higher temperatures due to the observed rate constant for homolysis being too fast in the presence of unlabelled substrate (Padmakumar *et al.* 1997).

The isotope effect on the first hydrogen transfer step (figure 1, step *ii*) has been examined under pre-steady-state conditions where the spectroscopic changes accompanying conversion of AdoCbl to cob(II)alamin were monitored (Padmakumar *et al.* 1997; Chowdhury & Banerjee 2000a,b). Surprisingly, the kinetics of the cobalt–carbon bond homolysis step were found to be sensitive to isotopic substitutions in the substrate (Padmakumar *et al.* 1997) and were interpreted as evidence for kinetic coupling between homolysis and the first hydrogen atom transfer step. A similar sensitivity of the rate of cobalt–carbon bond cleavage to isotopic substitution in the substrate has been seen in other B₁₂-dependent enzymes, including glutamate mutase (Marsh & Ballou 1998) and ethanolamine ammonia lyase (Bandarian & Reed 2000). Thus, it is postulated that in the absence of substrate, the equilibrium for the homolysis reaction favours the reverse reaction, i.e. geminate recombination of the deoxyadenosyl radical and cob(II)alamin (scheme 1, k_{-1}). Although the homolysis product, cob(II)alamin (Cbl• in scheme 1) is not observed by either EPR or UV-visible spectroscopy, the occurrence of cobalt–carbon bond homolysis under these conditions is supported by the observed stereochemical scrambling of deuterium introduced at C5' of AdoCbl (Gaudemer *et al.* 1981). In contrast, in the presence of substrate, a significant decrease in the energy barrier

Scheme 1.

concomitant with a change in the equilibrium constant for the homolysis reaction is postulated to favour forward propagation of the radical from the cofactor to the substrate (Chowdhury & Banerjee 2000a,b). Kinetic coupling explains why the detectable accumulation of cob(II)alamin is sensitive to the isotopic content of the substrate.

A detailed kinetic analysis of the substrate concentration and temperature dependence of k_{obsd} yielded the following thermodynamic parameters for the coupled homolysis/hydrogen atom abstraction step: $\Delta H^\ddagger = 18.8 \text{ kcal mol}^{-1}$, $\Delta S^\ddagger = 18.2 \text{ cal mol}^{-1} \text{ K}^{-1}$ and $\Delta G^\ddagger = 13.1 \text{ kcal mol}^{-1}$ at 37°C (Chowdhury & Banerjee 2000a,b). The corresponding values for thermolysis of AdoCbl in solution (a model for the uncatalysed homolytic cleavage of the cobalt–carbon bond) are: $\Delta H^\ddagger = 34.5 \text{ kcal mol}^{-1}$, $\Delta S^\ddagger = 14.0 \text{ cal mol}^{-1} \text{ K}^{-1}$ and $\Delta G^\ddagger = 30.2 \text{ kcal mol}^{-1}$. The enzymatic reaction is therefore characterized by a 17 kcal mol^{-1} decrease in the Gibbs free energy of activation, which corresponds to a 10^{12} -fold rate enhancement compared with the uncatalysed reaction.

Product release does not mask the KIE under presteady-state conditions. However, the k_{obsd} for the homolysis/hydrogen atom transfer step is a composite of multiple steps including minimally, substrate binding, protein conformational change, cobalt–carbon bond homolysis, deoxyadenosyl conformational change and hydrogen atom transfer from substrate. Among these, only the contribution of the substrate-binding step can be deconvoluted by analysis of the substrate concentration dependence of k_{obsd} (Chowdhury & Banerjee 2000a,b), the remainder are spectroscopically silent, at least under the conditions employed to monitor the reaction, i.e. detection of oxidation state changes in cobalamin. Furthermore, it is presumed but has not been demonstrated, that under pre-steady-state conditions, a single hydrogen transfer reaction occurs between the deoxyadenosyl radical and substrate. However, if this step is in rapid equilibrium and multiple hydrogen atom transfers occur on the experimental time-scale, the observed isotope effect will be a composite of a primary and an equilibrium isotope effect. It should be noted that since the methyl group of the substrate was fully deuterated in these experiments, the isotope effect is really a combination of primary and secondary effects. Owing to the relatively small contribution of the latter compared to the former, the contribution of the secondary isotope effect is omitted from this discussion.

Arrhenius analysis of the temperature dependence of the KIE under pre-steady-state conditions yields parameters that lie well outside the range expected for a semi-classical over-the-barrier process (Chowdhury & Banerjee 2000a,b). The slope of the line yields a difference in activation energy, $E_{\text{a,H}} - E_{\text{a,D}}$, of

$3.41 \pm 0.07 \text{ kcal mol}^{-1}$, while the intercept corresponds to the ratio of pre-exponential factors, $A_{\text{H}}/A_{\text{D}}$, of 0.078 ± 0.009 . The expected ranges for these values in the absence of tunnelling are: $A_{\text{H}}/A_{\text{D}} = 0.33\text{--}1.3$ and $E_{\text{a,H}} - E_{\text{a,D}} \leq 1.3 \text{ kcal mol}^{-1}$. The data from the Arrhenius analysis together with the measured deuterium KIE, which is significantly greater than the ground state zero-point energy maximum, are evidence for the involvement of tunnelling in the hydrogen atom transfer from the substrate to the deoxyadenosyl radical.

Thermolysis of AdoCbl or its analogues in protiated versus deuterated ethylene glycol has been studied as a model of the enzyme-catalysed homolysis and hydrogen atom transfer steps to the deoxyadenosyl radical (Doll & Finke 2003; Doll *et al.* 2003). Interestingly, these studies yield similar values for three parameters, the ‘Kreevoy criteria’ diagnostic of tunnelling (Kim & Kreevoy 1992); the magnitude of the KIE, the difference in the activation energies and the ratio of the Arrhenius pre-exponentials. Although the solution reaction is more appropriately a model for the B_{12} -dependent diol dehydratase reaction in which the deoxyadenosyl radical dehydratase abstracts a hydrogen atom from a diol substrate, it was argued that the extent of tunnelling in models for methylmalonyl-CoA mutase and diol dehydratase are unlikely to be significantly different based on the similar bond dissociation energies of the C–H bonds in the respective substrates (Doll *et al.* 2003). The correspondence in the magnitude of the ‘Kreevoy criteria’ parameters between the uncatalysed and methylmalonyl-CoA mutase-catalysed reactions has been interpreted as compelling experimental evidence against the hypothesis that enzymes have evolved to enhance the probability of quantum mechanical (QM) tunnelling, at least in B_{12} -dependent methylmalonyl-CoA mutase (Doll & Finke 2003; Doll *et al.* 2003).

3. COMPUTATIONAL ANALYSIS OF THE HOMOLYSIS REACTION

The size of the corrin ring, the presence of a transition metal and the radical character of the deoxyadenosyl moiety combine to make computational studies on methylmalonyl-CoA mutase, a challenging problem. Our own *N*-layered integrated molecular orbital and molecular mechanics (ONIOM) calculations using a recently developed optimization algorithm that allows the reaction coordinate to include atoms from both QM and molecular mechanics (MM) layers have permitted for the first time the calculation of the transition state (TS) for the enzyme-catalysed homolysis reaction (Kwiecien *et al.* 2006). Three models, based on the crystal structures of the enzyme in the presence and absence of substrate (Mancia *et al.* 1996, 1999; Mancia & Evans 1998), were employed in this

Figure 2. Computed classical mechanical potential of mean force for hydrogen transfer reaction between the deoxyadenosyl radical and methylmalonyl-CoA (reaction 2 in [scheme 1](#)) at (a) 5 °C and (b) 37 °C.

study. In the absence of substrate, the triosephosphate isomerase (TIM) barrel located above the corrin ring is flared open in a conformation that is referred to as the ‘open’ or resting state. The substrate binds in the cavity of the TIM barrel and triggers a large conformational change in which the barrel snaps in around the CoA-tail of the substrate. This is referred to as the ‘closed’ or active state. The third model represents the closed state from which the substrate has been subtracted (‘closed minus substrate’), a form that is not experimentally accessible, but is nevertheless potentially useful in querying the contribution of the closed conformation to activation of the cobalt–carbon bond.

In each model, the cofactor in the QM part comprised 71 atoms including the corrin ring without side chains, the ribose as the upper ligand and the imidazole as the lower ligand. The remaining part of the cofactor, the reactant in the ‘closed’ state model, and the active site residues within 15 Å of the cobalt were included in the MM part of the model. The resulting models contained 1372, 1339 and 1276 atoms in the ‘closed’, ‘closed minus substrate’ and ‘open’ states, respectively.

Relaxed potential energy scan (PES) calculations along the cobalt–carbon bond were carried out using the ONIOM protocol as implemented in GAUSSIAN03. The QM model of the truncated cofactor showed a smooth increase in energy that levelled off at *ca* 26 kcal mol^{−1}. Similar behaviour was observed for the ‘open’ and ‘closed minus substrate’ models although the maximum energy was lower in the latter and revealed a *ca* 12 kcal mol^{−1} stabilization of the cobalt–carbon bond by the substrate-induced protein conformational change. The PES for the ‘closed’ model revealed unexpected behaviour, and a sharp drop in energy was observed after reaching the maximum. This discontinuity indicated that the cobalt–carbon bond length *per se* is not a good approximation of the reaction coordinate for homolysis. A closer look at the atomic coordinates of this model as a function of increasing cobalt–carbon bond length indicated that the sudden drop in energy was paralleled by a concomitant change in the conformation of the deoxyadenosine, which is treated at the MM level in the model.

The TS was searched from the points on the PES, encompassing the maximum energy point and the

discontinuity point. The intrinsic reaction coordinate calculations confirmed the TS assignment. It is noteworthy that this represents the first successful attempt at finding a convergence to the TS for homolysis of AdoCbl. A saddle-point referred to as intermediate (I) was observed on the reactant side of the intrinsic reaction coordinate, which is *ca* 7 kcal mol^{−1} higher in energy than the starting structure. The TS for homolysis was only 3 kcal mol^{−1} higher than I and was exothermic by *ca* 7.5 kcal mol^{−1}. These calculations reveal that a significant degree of cobalt–carbon bond destabilization is achieved prior to homolysis and is accompanied by a marked change in the conformation of the deoxyadenosine moiety of AdoCbl, a lengthening of the cobalt–carbon bond and a rearrangement in the hydrogen-bonding interactions between the substrate and active site residues. The overall barrier for the homolysis step is *ca* 10 kcal mol^{−1} and the reaction is endothermic by *ca* 2.5 kcal mol^{−1}. The 5' carbon of the resulting deoxyadenosyl radical, the hydrogen atom donor in the subsequent step, is brought into van der Waals contact (3.226 Å) with the hydrogen atom donor, i.e. the methyl carbon of the substrate. The difference in the PESs for cobalt–carbon bond homolysis in free versus enzyme-bound AdoCbl in the presence of substrate points to the utilization of substrate-binding energy not only for catalysing this step, but also for positioning the resulting deoxyadenosyl radical for the subsequent hydrogen atom transfer step.

4. COMPUTATIONAL STUDIES ON THE ENERGETICS OF THE H-ATOM TRANSFER STEP

The two hydrogen atom transfer steps in the mutase reaction are the focus of this section. A minimum kinetic scheme for the methylmalonyl-CoA mutase-catalysed reaction is described by [scheme 1](#). In the step with the rate constants k_2 and k_{-2} , the transferred H originates in substrate and the deoxyadenosyl radical is the acceptor, whereas in the step with the rate constants k_4 and k_{-4} it derives from deoxyadenosine and is transferred to the product succinyl-CoA radical.

The PES for the hydrogen transfer reactions catalysed by methylmalonyl-CoA mutase were modelled using molecular dynamics umbrella sampling simulation and by employing a combined generalized

Figure 3. Computed classical mechanical PMF at 5 °C for hydrogen transfer reaction between the succinyl radical and deoxyadenosine (reaction 4 in scheme 1).

hybrid orbital QM/MM (GHO-QM/MM) method (Gao *et al.* 1998; Truhlar *et al.* 2002). The QM subsystem was treated by the Austin Model 1 (AM1) method. To average the dynamical effects over the ensemble of possible conformations of TSs along the reaction coordinate, the potential of mean force (PMF; Valleau & Torrie 1977) was computed. Thirteen and 19 windows were sufficient to cover the entire reaction path for hydrogen atom abstraction by the deoxyadenosyl (reaction 2) and the succinyl (reaction 4) radicals, respectively. The classical mechanical PMFs determined on their basis are shown in figures 2 and 3. The free energies of activation obtained at different levels of theory (classical, with inclusion of quantized vibrational contributions and with dynamical recrossing and multidimensional tunnelling) are presented in table 1.

Comparison of figure 2*a,b* reveals an interesting behaviour of the hydrogen transfer step, which changes from being thermoneutral at 5 °C to slightly exothermic (by approx. 3 kcal mol⁻¹) at 37 °C. While it is difficult to validate the computed energetic results, support for the data in table 1 comes from preliminary calculations of classical barriers for the model systems that were performed at a number of theoretical levels (Dybala-Defratyka *et al.* 2004). As can be seen from the data in table 2, semi-empirical AM1 values parallel to those obtained at the density functional theory (DFT) level are used for barrier height calculations (Lynch & Truhlar 2003) and agree with the value of 16.04 kcal mol⁻¹ evaluated from high-end calculations for the Pr• + Pr reaction. Thus, the use of the AM1 method for describing the quantum part in the QM/MM calculations is justified by these results.

The free energy of activation computed for the first hydrogen abstraction step (reaction 2) revealed interesting information as compared with the experimentally observed value. First, the experimental value of ca 13 kcal mol⁻¹ measured at 310 K (Chowdhury & Banerjee 2000*a,b*) is expected to be slightly higher at 278 K, the temperature at which the simulations were conducted. As the temperature decreases, the rate of the reaction also decreases. This would lead to an increase in the barrier height by approximately 1–2 kcal mol⁻¹ at 278 K. Based on this extrapolation,

Table 1. Calculated free energies of activation ($\Delta G^\ddagger$, kcal mol⁻¹) for four hydrogen transfer reactions.

reaction	temperature (°C)	barrier		
		classical	with quantized vibrations	with recrossing and tunneling
2/–2	5	21.3	19.1	16.6
2	37	20.8	18.3	~15.8 ^{a,b}
–2	37	23.8	21.3	~18.8
4	5	22.3	20.1	~17.6 ^b
–4	5	14.0	11.9	~9.4 ^b

^a This value can be compared with the experimental 13.1 ± 0.6 (s.d.).

^b This is based on the assumption that in these cases recrossing and tunnelling also lower the energy by 2.5 kcal mol⁻¹.

Table 2. Classical barriers for chemical models.

	Pr + Pr ^a (kcal mol ⁻¹)	iPr ^a + Pr (kcal mol ⁻¹)
AM1	15.6	19.0
MPW1K	17.0	20.4

^a Pr, propyl; iPr, isopropyl.

the experimental free energy of activation is estimated to be ca 15–16 kcal mol⁻¹ at 278 K, which is in good agreement with the calculated value from this study (16.6 kcal mol⁻¹). Preliminary data on the homolysis step (reaction 1) estimated an energetic barrier for this step of ca 10 kcal mol⁻¹ (298 K, ΔE) and a reaction endothermicity of ca 2.5 kcal mol⁻¹. This yields a lower barrier for the recombination reaction (scheme 1, reaction 1), which along with the barrier for the reaction 2 reported here, predicts the following kinetics. The apparent KIE_{exp} for the stepwise reaction (scheme 1) can be expressed by:

$$\text{KIE}_{\text{exp}} = \frac{\text{KIE}_{\text{int}} + k_2/k_{-1}}{1 + k_2/k_{-1}}, \quad (4.1)$$

where KIE_{int} corresponds to the KIE on the rate constant k_2 (called the intrinsic KIE). The assumption made in deriving this equation is that only the step with the rate constant k_2 (reaction 2 of scheme 1) is isotope sensitive (Paneth 1985). This assumption is justified in this case because homolytic cleavage of the cobalt–carbon bond does not involve hydrogen atoms. By analogy, the same reasoning is applied for the rate constants k_4 and k_{-4} . The energetic results obtained for the initial steps of the methylmalonyl-CoA mutase-catalysed reaction suggest that reaction 2 is slower than reaction 1. Hence according to equation (4.1), the intrinsic KIE should be fully expressed and equal to the observed value. If this is indeed the case, the KIE computed at 278 K is predicted to be ca 50, i.e. k_2/k_{-1} in equation (4.1) equals zero (Chowdhury & Banerjee 2000*a,b*). Preliminary data confirm this prediction (A. Dybala-Defratyka *et al.* 2006, unpublished results).

Another intriguing step in the mutase-catalysed reaction is the H atom abstraction from succinyl-CoA

Figure 4. Qualitative free energy profile of the methylmalonyl-CoA mutase-catalysed reaction. The substrate-binding step is omitted. Steps 2 and 4 have been examined in this study, whereas 1, 3 and 5 are derived from elsewhere (1, Kwiecien *et al.* (2006); 3, Loferer *et al.* (2003) and 5, Kwiecien & Paneth (2006, unpublished results).

(reaction 4). Our results show that the free energy of activation of the hydrogen atom transfer in the presence of succinyl-CoA (reaction –4) is significantly lower than with methylmalonyl-CoA (reaction 2) and is equal to *ca* 10 kcal mol^{–1} or may even be lower if the tunnelling contribution is higher than we estimated. Thus, assuming that the cobalt–carbon bond ruptures in the presence of succinyl-CoA in the same fashion as it does in the presence of methylmalonyl-CoA, this would result in the rate constant for this step (reaction 4) being similar to the recombination (reaction 5). This in turn would lead to the KIE for the first hydrogen atom transfer step in the reverse direction being significantly reduced.

On the basis of the available information from kinetic (Chowdhury & Banerjee 2000*a,b*), EPR (Mansoorabadi *et al.* 2005) and modelling (Loferer *et al.* 2003) studies, we propose the qualitative free energy profile shown in figure 4 for the methylmalonyl-CoA mutase-catalysed reaction that includes all steps excluding substrate binding.

5. CONCLUSIONS

Computational studies on the cobalt–carbon bond homolysis and hydrogen atom transfer steps are furnishing needed insights into structural reorganizations that accompany the chemical steps. They are also providing energetic estimates of the component steps that are experimentally intractable, being spectroscopically silent. The results of some of the computational studies, viz. changes in the hydrogen-bonding network to the deoxyadenosine and substrate during homolysis and the predicted lack of an isotope effect in the reverse reaction under presteady-state conditions, are amenable to experimental validation. The complementary experimental and computational approaches for examining an issue of long-standing debate, the labilization of the cobalt–carbon bond by B₁₂-dependent isomerases and the sensitivity of this step to isotopic substitution in the substrate in methylmalonyl-CoA mutase, are beginning to yield interesting mechanistic insights.

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