

Supporting Information

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SI Text

All *ab initio* calculations were performed with the Gaussian 03 software (1). Density functional theory (DFT) calculations were done using the hybrid Becke three parameter functional (2) with the correlation functionals of Lee, Yang, and Parr (3) and Vosko, Wilk and Nusair (4) in the assembly known as B3LYP (5, 6). Explicit valence electron correlation was computed with frozen core Møller-Plesset perturbation theory to the second order (MP2) (7) and we used Pople's split valence basis sets 6-31G, 6-31G* and 6-311G** (8–10) augmented with diffuse and polarized functions (11). If nothing else is stated, B3LYP is short for B3LYP/6-311G**, B3LYP** stands for B3LYP/6-311++G(2df,pd) with tight SCF convergence criterion and MP2 similarly refers to FCMP2/6-311G**. Geometry optimizations were done either with the direct inversion in the iterative subspace (GDIIS) method (12) or the Bery algorithm by Schlegel and colleagues (13) using the standard force and displacement convergence criteria throughout.

The initial positions of the model systems were taken from a representative MD snapshot of the tetrahedral intermediate state as given by our previous EVB simulations (14, 15). These initial positions were gradually relaxed with partial GDIIS optimizations at the B3LYP/6-31G* level where some initial positional constraints were applied keep the cluster intact. Starting from this partially relaxed structure Bery calculated the ground state potential surface by varying the inter-nuclear distances of the reacting atoms in two consecutive parts. In the initial scan the $r(\text{O}3', \text{carbonyl C})$ distance was incremented from 1.5 to 2.5 Å and the $r(\text{O}2', \text{amino H})$ distance was decremented from 1.8–1.0 Å in five steps each (grid shown in Fig. 3A). From these 25 structures, 10 were extracted for the next round of scans: the start, end and mid-point distances (i.e., a 3 x 3 grid), as well as the saddle point structure found at $r(\text{O}3', \text{carbonyl C})$ equals 1.8 Å and $r(\text{O}2', \text{amino H})$ equals 1.2 Å (encircled in Fig. 3A). In these scans the hydrogen distances from the 2'-hydroxyl to the water bridge oxygen and the water bridge hydrogen to the O3' were varied between 1.6–1.0 and 0.9 Å respectively in five steps each. Additional constraints were imposed to speed up the geometrical convergence of these scans. The hydrogen scans did not change the qualitative behavior of the initial scan and the approximate transition state structure was found in the scan grid based on the saddle point structure, specifically at $r(\text{H}2', \text{water O})$ equals 1.2 Å and $r(\text{O}3', \text{water H})$ equals 1.5 Å.

The approximate eight-membered transition state structure from these 265 geometry optimizations was used as the input guess in a constrained transition state optimization with the synchronous transit-guided quasi-Newton method (STQN) (13). Once found, the constraints in this structure were gradually released while keeping the reacting atoms frozen. Bery then partially optimized the resulting structure to a TS followed by another gradual release, a procedure that was iterated until the constraints were all but removed. To unfreeze the last coordinates and reach a true minimum of the ground state potential yet another water molecule was added to the model. This water molecule stabilizes the C2063 2'-hydroxyl and its complexed water molecule by forming a hydrogen bond to the O3' (as can be seen in e.g., Fig. S1). However, there seems to be plenty of space available on the ribosome for such a water molecule judging from crystallographic data (16, 17) and solvated MD simulations (14, 15). The resulting unconstrained transition state structure was used as an input guess for a higher level TS optimization (B3LYP/6-311G**), which converged to the structure shown in Fig. 2A.

A six-membered TS was optimized in a similar fashion. It was found that a stable tetrahedral intermediate requires two screening water molecules on the carbonyl oxygen (the "oxyanion") to form. However, these two models only include one such water molecule, and thus have a transient TI, making the optimizations more complicated. In the previous model a stable TI was formed using peripheral constraints, whereas this optimization started with a scan of the distance between the attacking amino nitrogen and carbonyl carbon in order to locate its saddle point. Then this structure was subjected to a partial QST2 optimization to find the TS, after which the constraints were gradually relaxed in an iterative fashion as above. The resulting fully relaxed TS was then optimized to the B3LYP/6-311G** level and is shown in Fig. 4A and Fig. S1. The eight-membered structure was used as a starting guess for the optimization of transition states in absence of an oxyanion bound water molecule and with the addition of an extra screening water molecule.

These four TS structures were validated with frequency calculations and then intrinsic reaction coordinate (IRC) (18) following by the method of Gonzalez and Schlegel (19, 20). Each TS showed one imaginary frequency vibrating in the direction of the reaction coordinate at 920, 902, 556, and 121 cm^{-1} for the six- and eight-membered transition states with no, one, and two screening oxyanion waters, respectively. The six-membered IRC was followed for 120 steps, or 12 Å, producing as many structures, and the single screening water eight-membered TS for 210 steps, or 20 Å, as shown in Fig. 3B–C. The IRCs were seen to connect the transition states with reactants and products but were not followed all the way down to their corresponding potential wells. Instead unconstrained reactant structures were carefully optimized from the end points of these IRCs, using similar iterative partial optimization methods as outlined above. Since the IRCs of the standard six and eight-membered transition states were seen to be well behaved, the IRCs of the naked and doubly screened oxyanion were followed for only six steps in the forward and reverse direction.

To gauge the effect of explicit electron correlation in these systems, MP2 single point energies were computed along the IRC. In the spirit of IRCMax theory (21) the maximum of such a higher level calculation along the IRC will correspond to the TS structure optimized at the higher level. Whereas it is known that B3LYP structures perform extremely well in the space perpendicular to the reaction coordinate (22), the semilocal hybrid B3LYP functional is not expected to give accurate energies along the reaction coordinate because of strong correlation upon bond stretching as discussed in, e.g., (23). IRCMax theory addresses this issue by effectively optimizing at a higher level along the IRC, i.e. in the degree of freedom where bonds are broken and remade. Because we opt for MP2 in the high level calculations these results are independent of the choice of DFT functional along the reaction coordinate. The computed MP2 energies along the IRC are shown in Fig. 3B–C. The DFT structures are seen to be correct to the IRCMax{MP2/6-311G**//B3LYP/6-311G**} level, except in the case of the doubly screened oxyanion eight-membered TS where it is found 0.5 Å further along the IRC closer to the product state. However, the 0.6 kcal/mol increase in electronic energy is overshadowed by a 4 kcal/mol drop in enthalpy (see Table S1) which favors the MP2//B3LYP solution at the B3LYP TS in this case. It is noteworthy that explicit valence electron correlation shifts the barriers 8 kcal/mol closer to the experimental barriers. Whether this is due to a factual improvement by MP2 or a possible

underestimation of the barriers could, in principle, be examined by rigorous coupled cluster theory. Although the hybrid B3LYP functional appears to overestimate the barrier for this complicated concerted reaction path, semilocal DFT is often found to underestimate reaction barriers and has been shown to give too low energies at infinite fragment separation (24, 25).

The vibrational contribution to the enthalpy depends on an accurate description of the harmonic frequencies in these systems. It has been shown that B3LYP/6-311G** approaches QCISD accuracy when using scaled (0.99) calculated harmonic constants, a result that does not improve much if the basis set is extended (22). Projected frequencies (26) were computed along the IRCs to estimate the enthalpy barriers at 298 K (shown in Fig. 3B–C, unscaled frequencies). Their maxima are seen to roughly correspond to the optimized transition states (i.e., the saddle points of the potential energy surfaces) although their jagged appearance reveals uncertainties in the underlying IRC structures (cf. figures 3–5 in ref. 26). When computing enthalpies the frequencies were kept unscaled since this is not a significant source of error (approximately 0.1 kcal/mol). Low frequencies, i.e., modes for which about 5% of the ensemble population are likely to be in an excited vibrational state at room temperature, are of greater concern here for two reasons: They correspond to intermolecular vibrations that we wish to include (27), but they are at the same time confounded by internal rotations (e.g., spinning methyl groups) which can introduce significant error in the thermochemistry when the harmonic approximation is used. Low frequencies have been included here in the harmonic approximation and contributions from internal rotations are expected to cancel between the TS and reactant states.

The final reaction barriers with respect to the enthalpy were estimated by combining the MP2 barriers and DFT vibrational enthalpies with a high-level basis set correction as shown in Eq. S1. The relative contribution from each term in this composition is found in Table S1. In this formula and table δH is the correction to the vibrational enthalpy at 298 K and $\Delta\delta H^\ddagger$ is the corresponding correction to the activation enthalpy. Unless stated otherwise the sign[‡] refers to the IRCMax MP2 level TS structures.

$$\begin{aligned}\Delta H_{calc}^\ddagger &= B3LYP^\ddagger - B3LYP^R + \delta H_{B3LYP}^\ddagger - \delta H_{B3LYP}^R \\ &+ \Delta(MP2 - B3LYP)^\ddagger, R + \Delta(B3LYP^{++} - B3LYP)^\ddagger, R \\ &= \Delta B3LYP^\ddagger + \Delta\delta H_{B3LYP}^\ddagger + \Delta\Delta MP2^\ddagger + \Delta\Delta B3LYP^{++\ddagger}\end{aligned}\quad [S1]$$

Furthermore, ¹⁴N/¹⁵N, ¹⁶O/¹⁸O and ¹²C/¹³C kinetic isotope effects (KIE) were calculated at 310 K with the Bigeleisen-Wolfsberg equation as implemented in the IsoEff98 software by Anisimov and Paneth (28). The reactant and TS frequencies were scaled by 0.99 in this calculation (*vide supra*). Finally, natural bond orbitals (NBO) (29, 30) and natural population analyses (NPA) (31) were evaluated on the high level B3LYP⁺⁺ densities in all reported cases.

It has been hypothesized that there exists a very early transition state in the peptidyl transfer reaction where the N–C distance is large (32). However, a scan comprising 625 partial geometry optimization for a six-membered model (Fig. S3) shows that this region of the energy surface does not contain any such transition state. The only saddle point in this region is the transient tetrahedral intermediate which is the starting point of the scans in Fig. 3. The reported quantum mechanical calculations in ref. 32, for the approach of an ammonia molecule towards formic acid, found a small barrier of 1.6 kcal/mol at an N–C distance of 2.0 Å, yielding a normal ¹⁴N/¹⁵N KIE. However, this is not a TS of the actual reaction since neither C–O bond fission nor proton transfers occur. Fig. S3 shows the energy surface and reaction coordinate derivatives at this N–C distance and it is clear that the six-membered mechanism does not have a TS in this region. Why the experimentally measured ¹⁴N/¹⁵N KIE was found to be normal (32) is somewhat obscure in view of the present results. However, it should perhaps be pointed out that 50S assay with a CC-puromycin derivative (CCA_N-Phe) was very slow and, e.g., showed only a small effect of substituting the attacking amine for a hydroxyl group, in contrast to the almost four order of magnitude effect found by Rodnina and coworkers for full-length tRNAs (33).

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Table S1. Decomposition of the different contributions to the calculated activation enthalpy as given by Eq. S1. Also given are the corresponding summations when the vibrational enthalpies have been exchanged with the zero-point energies. The bottom row gives the vibrational contribution to the entropy term. All values at 298 K.

| | <i>Six mem.</i> | <i>Eight-membered</i> | | | |
|-----------------------------------|-----------------|-----------------------|-----------------------|---------------------------------------|-------------|
| | | O=C< | H ₂ O-O=C< | (H ₂ O) ₂ -O=C< | |
| | | | | MP2//B3LYP | IRCMax |
| $\Delta B3LYP^\ddagger$ | 38.0 | 29.6 | 29.5 | 26.4 | 26.5 |
| $\Delta\Delta H_{vib}^\ddagger$ | -3.5 | -4.6 | -4.6 | -3.2 | -7.2 |
| $\Delta\Delta MP2^\ddagger$ | -7.6 | -4.8 | -7.9 | -12.0 | -11.6 |
| $\Delta\Delta B3LYP^{++\ddagger}$ | 2.3 | 1.2 | 1.5 | 2.9 | 3.0 |
| Sum | 29.3 | 21.3 | 18.5 | 14.1 | 10.7 |
| $\Delta\Delta ZPE$ | -2.1 | -3.2 | -3.2 | -0.8 | -5.1 |
| Sum | 30.7 | 22.8 | 19.9 | 16.5 | 12.8 |
| $-T\Delta S_{vib}^\ddagger$ | 5.8 | 4.0 | 4.3 | 7.3 | |