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The α -Amino Group of the Threonine Substrate As The General Base During tRNA Aminoacylation: A New Version of Substrate Assisted Catalysis Predicted by Hybrid DFT

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Abstract

Density functional theory-based methods in combination with large chemical models have been used to investigate the mechanism of the second half-reaction catalyzed by Thr-tRNA synthetase; aminoacyl transfer from Thr-AMP onto the $A_{76}3'OH$ of the cognate tRNA. In particular, we have examined pathways in which an active site His309 residue is either protonated or neutral (i.e., potentially able to act as a base). In the protonated His309-assisted mechanism, the rate-limiting step is formation of the tetrahedral intermediate. The barrier for this step is 155.0 kJ mol⁻¹ and thus, such a pathway is concluded to not be enzymatically feasible. For the neutral His309-assisted mechanism two models were used with the difference being whether Lys465 was included. For either model the barrier of the rate-limiting step is below the upper-thermodynamic enzymatic limit of ~125 kJ mol⁻¹. Specifically, without Lys465 the rate-limiting barrier is 122.1 kJ mol⁻¹ and corresponds to a rotation about the tetrahedral intermediates C_{carb}—OH bond. For the model with Lys465 the rate-limiting barrier is slightly lower and corresponds to the formation of the tetrahedral intermediate. Importantly, for both neutral His309' models the neutral amino group of the threonyl substrate directly acts as the proton acceptor; in the formation of the tetrahedral intermediate the $A_{76}3'OH$ proton is directly transferred onto the Thr-NH₂. Therefore, the overall mechanism follows a general substrate assisted catalytic mechanism.

1. Introduction

Proteins are the 'work horses' of cells being involved in almost all aspects of their physiology. Typically, their synthesis within cells occurs via a multi-step process of transcription and translation (i.e. initiation, elongation and termination).¹ However, prior to their inclusion in an elongating protein, the intended constituent amino acids must first be attached to their cognate tRNA.^{2–4} This process occurs via two half-reactions that are catalysed by the class of enzymes known as aminoacyl-tRNA synthetases (aaRS's).^{2–6}

The first half-reaction is activation of the amino acid by reacting it with adenosine triphosphate (ATP) to give the corresponding aminoacyl-adenylate (aa-AMP) plus

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SUPPORTING INFORMATION AVAILABLE:

Cartesian coordinates of the optimized structures reported in this manuscript. Full citation of the Gaussian 09 program. This information is available free of charge via the Internet at <http://pubs.acs.org>.

pyrophosphate (PPi). This reaction has previously been studied both experimentally and computationally in detail.^{6–14}

In the second-half reaction the aminoacyl moiety is transferred from the aa-AMP onto its cognate tRNA to give the desired aminoacyl-tRNA product.^{2–6} Remarkably, this reaction is catalysed by the same aaRS as for the first half-reaction. More specifically, the 2' (class I aaRS's) or 3'OH (most class II aaRS's) oxygen of the A76 residue of the tRNA substrate nucleophilically attacks the carbonyl carbon of the aa-AMP co-substrate.^{4,7,15,16} That is, it attacks the amino acid residue's carboxylic carbon.^{2,3} This ultimately results in cleavage of the phosphoester link in aa-AMP with formation of an A76-(2' or 3')O—C(O)R ester bond; i.e., transfer of the aminoacyl moiety onto the A76 residue of the cognate tRNA.^{16,17} It is generally held that aaRS's utilize a base catalysed mechanism in which an appropriate basic active site group deprotonates the nucleophilic A76 hydroxyl group.^{17–19} However, the identity of the actual mechanistic base is often unclear.

Experimentally, X-ray crystal structures have been obtained for class I GlnRS by Perona et al.,¹⁷ and several class II aaRS's including HisRS by Guth et al.,¹⁶ and AspRS by Eiler et al.^{18–20} In each structure it was observed that a non-bridging phosphate oxygen of the aminoacyl-adenylate substrate was in close proximity to the sugar hydroxyl of the tRNA cosubstrate's A76 to which the aminoacyl moiety is to be transferred. In addition, Francklyn and coworkers¹⁶ performed a detailed experimental mutagenesis study on a non-metal containing HisRS. They observed that substitution of the *pro-R* (O_{pro-R}) and *pro-S* (O_{pro-S}) non-bridging aa-AMP phosphate oxygens by sulfur resulted in a 50- and 10000-fold decrease in the rate of reaction, respectively.^{16,21} Shortly thereafter, Perona et al. performed a mutational analysis on GlnRS and concluded that it followed the same mechanism.²² Thus, it was proposed that in the catalytic mechanism of HisRS O_{pro-S} acted as a general base and deprotonated the hydroxyl of the A76 residue of the tRNA cosubstrate as shown in Scheme 1.¹⁶ Based on their observed dissimilar active sites and apparent common utilization of a non-bridging phospho-oxygen of the substrate as the mechanistic base, it has been suggested that for aaRS's such a substrate-assisted catalysis (SAC) mechanism may be a general feature of these presumably ancient enzymes.^{5,16,22–26}

Following these experimental studies we performed a detailed density functional theory (DFT)-based computational investigation on the catalytic mechanism of HisRS.⁵ In particular, the ability of the aa-AMP's bridging and *pro-R* and *pro-S* non-bridging oxygens to act as a base and the mechanism by which they may catalyse the aminoacylation reaction was systematically examined. It was found that the O_{pro-S} oxygen could indeed act as the required base. Furthermore, in the resulting stepwise reaction pathway the rate limiting step was the initial proton transfer from the 3'OH group to O_{pro-S} with a barrier of approximately 109.2 kJ mol^{–1} (see Scheme 1). It was also noted that the tetrahedral intermediate was only stable with respect to conversion to the reactants or products by less than 5 kJ mol^{–1}. Thus, *in vivo*, the overall mechanism may effectively be concerted as had been experimentally suggested.^{16,17}

Recently, there have been several experimental studies on a metal containing ThrRS.^{21,27–29} In contrast to that observed for HisRS,¹⁶ mutagenesis studies by Francklyn and coworkers found that substitution of either non-bridging phosphate oxygens of the threonyl-adenylate substrate by sulfur resulted in only quite minor, three-fold or less, decreases in the rate of reaction.²¹ However, substitution of a histidyl residue (His309),²¹ thought to be positioned near the A76 2'OH group, decreased both k_{cat} (aminoacylation) and k_{trans} (aminoacyl transfer) by 34- and 242-fold, respectively. Consequently, a catalytic mechanism for ThrRS was proposed in which the His309 residue plays the role of the mechanistic base as shown in Scheme 2.^{21,28} Specifically, it acts by deprotonating the 2'-OH group of the tRNA's A76

residue. It was further suggested that this may occur directly or indirectly via a bridging water molecule between the 2'OH group and His309. The resulting 2'O⁻ then deprotonates the adjacent 3'OH group enabling the now negatively charged 3'O to act as a nucleophile and attack the carboxylic carbon of the threonyl-adenylate co-substrate, leading to transfer of the aminoacyl moiety.

It is noted that the active site of ThrRS also contains a Zn(II) ion coordinated to the enzyme via two histidines (His385 and 511) and a cysteine (Cys334) residue.^{21,27,30} X-ray crystal structures^{27,30–33} have been obtained of ThrRS with and without various substrate analogues bound within the active site. Based on the observed differences it has been suggested that the role of the Zn(II) may simply bind the substrate, possibly with displacement of a Zn(II)-bound water.³⁰ Indeed, in all available X-ray crystal structures of ThrRS bound with threonine or a threonyl-AMP analogue such as those obtained by Torres-Larios et al.,³³ Sankaranarayanan et al.,³² and Dock-Bregeon et al.,³⁰ the threonyl or its analogue are bidentately ligate via their R-group hydroxyl and neutral α -amino group to the Zn(II) ion. In fact, it has been suggested that this bidentate coordination of the threonyl to the Zn(II) is an essential characteristic of the active site of ThrRS that allows the enzyme to discriminate against the isosteric valine.³³

In this present study we have investigated the aminoacyl transfer mechanism as catalysed by threonyl-RNA synthetase using density functional theory-based methods in combination with large chemical models. For completeness and to provide additional insights into the roles of active site residues and functional group moieties, we have examined the ability of the active site His309 residue to act as either a catalytic acid or base.

2. Computational Methods

All calculations were performed using the Gaussian 09 program³⁴ and employed the hybrid density functional theory B3LYP method.^{35–37} Optimized geometries of all species detailed herein were obtained at the B3LYP/6–31G(d,p) level of theory as were their corresponding harmonic vibrational frequencies in order to confirm the nature of the stationary points, unless otherwise noted. In the “neutral His309” mechanism the pathway connectivity of all transition structures was confirmed by IRC calculations. The general effects of the surrounding polar protein environment were included via the integral equation formalism polarizable continuum model (IEF-PCM)³⁸ with a dielectric constant (ϵ) of 4.0, a value commonly used for proteins.³⁹ Relative energies were obtained by performing single point calculations at the IEF-PCM(ϵ =4.0)-B3LYP/6–311+G(2df,p) level of theory on the above optimized structures.

The two active site chemical models used in this present study were derived from our previous MD study⁴⁰ on the fully bound active site. While the full details of this study are not repeated herein, those most pertinent to the present model selection are briefly summarized. In particular, the MD simulations examined the structure of the fully bound enzyme-ThrAMP/ThrRNA complex. These were based in part on the experimental²⁷ X-ray crystal structure (PDB: 1QF6) in which both AMP and ThrRNA are bound within the active site while the threonyl moiety was added in accordance with several available X-ray crystal structures containing threonyl or similar groups.^{30,32,33} The resulting complex was then solvated and annealed for 500 ps followed by 10 ns production simulations in which the His309 residue and ThrAMP's α -NH₂ group was either neutral or protonated. It was concluded that the most likely initial states of the bound active site have a neutral α -NH₂ group and either a neutral or protonated (His309-H⁺) residue. A clustered based approach was then used with the distance between the attacking α -C'OH (H) and carboxylic carbon (C) of the threonyl chosen as the clustering parameter. The average structure from the most

populated cluster for each simulation was then retained, and then minimized using the AMBER99 MM method. The optimized structures were then used to derive the bound active site models used in the present hybrid DFT investigations and shown in Scheme 3. The key active site residues and substrates were modeled as follows: Thr-AMP by methyl monophosphate threonyl; A76 of the cognate tRNA by 1',5'-deoxyribose; Arg363 by ethylguanidinium cation; Gln484 acetamide; Glu383 by acetate; His385 and His511 by imidazole; and Cys334 by methylthiolate. In case of His309 it was modeled as either neutral or protonated 1-ethyl-imidazole. In addition, the Zn(II) cation and an active site water were also included. It is noted that the latter has been observed within the X-ray crystal structures.²⁷ Selected atoms in the models were fixed at their final MM minimized positions in order to maintain integrity in the spatial arrangement of active site residues (Scheme 3). This computational approach has been successfully applied to related enzymes and has been previously reviewed in detail.^{39,41–44} It has been noted that this approach can commonly gives errors of approximately 12 kJ mol⁻¹.³⁹

In both bound active site models employed herein the threonyl's α -amino group (Thr-NH₂) has been modeled as initially neutral (i.e., -NH₂). This is due to the fact that, as previously noted in the Introduction, in all available X-ray crystal structures^{30,32,33} in which a threonine or threonyl-AMP analogue is bound within the active site of ThrRS, the threonyl moiety at least initially coordinates via its R-group hydroxyl and α -NH₂ group to the Zn(II) centre. In addition, in most class II aaRS it has been observed that the substrates α -amino group forms an ion pair with an active site glutamyl or aspartyl residue.^{16,20,45,46} However, such an interaction is absent in ThrRS.²¹ Rather, the Thr-NH₂ group does lie near the side chain of Tyr462, which is itself hydrogen bonded to A762'O, Gln484, as well as those of several hydrophobic residues. Furthermore, in a previous QM/MM computational investigation on the first half-reaction as catalyzed by ThrRS, Zurek et al.⁸ observed that if the threonyl's α -amino was protonated, the threonyl substrate rotated within the active site such that its α -carboxylate instead ligated to the Zn(II) ion.⁸ It should also be noted that we did optimize the structure of the fully bound active site (using the method detailed above) in which the threonyl's α -amino group was modeled as protonated (i.e., -NH₃⁺). However, it was found that the -NH₃⁺ group shifted away from the Zn(II) ion and instead formed a strong hydrogen bond with A763'O.

In addition, a topological analysis of the electron densities at selected bond critical points was performed on the above optimized structures at the B3LYP/6–31G(d) level of theory using the AIM2000 program.⁴⁷

3. Results and Discussion

3.1. Substrate-bound active site

We began by considering the initial fully bound active sites in which the His309 residue is either protonated (His309–H⁺) and thus may potentially act as an acid, or neutral and thus may act as a base. For both systems, two complexes lying close in energy were obtained: a 'pre-reactive' complex (**PRC**) in which the threonyl's α -NH₂ group is coordinated to the Zn(II) centre, which is consequently five-coordinate; and a 'reactive' complex in which the same α -NH₂ is no longer ligated to the Zn(II) centre, which is, as a result, four-coordinate. It is noted that unlike that observed in some aaRS's,^{16,48} in ThrRS there is no adjacent residue carboxylate with which the threonyl's α -amino can interact.

'Pre-reactive' complexes (**PRC**'s)

The optimized structures, with selected bond and interaction distances of the PRC's obtained in which His309 is protonated (**^aPRC**) or neutral (**^bPRC**) are shown in Figure 1. Both

overall structures are in reasonable agreement with experimentally obtained X-ray crystal structures. For example, in both complexes one of the substrates non-bridging phosphate oxygens binds to the guanidinium of an arginyl (Arg363) in agreement with that observed²⁷ experimentally. Furthermore, the threonyl moiety coordinates to the Zn(II) ion via both its R-group hydroxyl (Thr-OH) oxygen and -NH₂ nitrogen (N_{Thr}) centres. More specifically, the Thr-OH oxygen forms a short strong interaction with the Zn(II) centre with Zn...O(H)_{Thr} distances of 1.98 and 1.99 Å in **aPRC** and **bPRC**, respectively. This is likely due in part to the fact that it is also simultaneously hydrogen bonded to the carboxylate of an adjacent aspartate (Asp383), thus enhancing the nucleophilicity of the Thr-OH oxygen (Figure 1). The Zn...N_{Thr} coordination bonds in **aPRC** and **bPRC** are both slightly longer at 2.24 and 2.28 Å, respectively. Thus, as noted above, the Zn(II) centre in the PRC's is five-coordinate as it is also ligated to the enzyme via two histidines (His511 and His385) and a cysteine (Cys334). Both optimized structures are in agreement with X-ray crystal structure of, for example, a bound ThrRS...SerAMP complex, which has a similar but slightly longer Zn...N_{Thr} distance of 2.33 Å.

'Reactive' complexes (RC's)

Notably, alternate substrate-bound active site complexes were also obtained in which the threonyl moiety is *only* coordinated to the Zn(II) centre via its R-group oxygen; that is the Zn...N_{Thr} coordination bond has been cleaved and the Zn(II) centre is now, as a result, four-coordinate. The optimized structures with selected bond lengths of the corresponding resultant reactive complexes, **aRC** and **bRC** respectively, are shown in Figure 1. Importantly, these complexes lie only marginally higher in energy than their corresponding pre-reactive complexes **aPRC** and **bPRC** by 13.6 and 7.5 kJ mol⁻¹, respectively. Thus, similar to that experimentally observed for other Zn(II)-containing complexes,^{49–52} the Zn...N_{Thr} coordination bond appears to be quite labile with the Zn(II) center able to easily interconvert between five and four coordinate with little cost in energy (see below).

In both **aRC** and **bRC** the distance between the Zn(II) ion and the threonyl's amino nitrogen (N_{Thr}) centre has lengthened markedly with *r*(Zn...N_{Thr}) distances of 3.07 and 3.43 Å, respectively. In fact, in both cases the N_{Thr} centre now forms a moderately strong hydrogen bond (H-bond) with the A763'OH of the tRNA cosubstrate with ThrN...HO3' distances of 1.91 and 1.94 Å respectively. The properties of the density at the bond critical point (BCP) of a particular interaction can give additional insights. For hydrogen bonds (H-bonds), the typical values of the electron density (ρ) and its Laplacian (∇^2) are said to be 0.002–0.040 and 0.024–0.139 respectively.^{53,54} As can be seen from the results of the topological analyses of **aRC** and **bRC** given in Table 1, these two ThrN...HO3' interactions are calculated to have very similar ρ values of 0.034 and 0.033 that are towards the upper end of the range typically noted for such bonds (i.e., are relatively strong H-bonds).^{53,54} In addition, in **aRC** and **bRC** the crystallographically observed active site H₂O now hydrogen bonds to the threonyl's carbonyl oxygen (C_{carb}=O) with C_{carb}O...HOH distances of 1.92 and 1.83 Å, respectively. The comparative strength of these two interactions is reflected in their BCP ρ and ∇^2 values of 0.026 and 0.073, and 0.032 and 0.097 respectively, i.e., the C_{carb}O...HOH H-bond in **bRC** is stronger.

As a result of the dissociation of the Zn...N_{Thr} coordination bond, the threonyl's R-group hydroxyl oxygen is now more strongly ligated to the Zn(II) ion than in the corresponding **PRC**'s. Hence, the Zn...O(H)_{Thr} distances are now markedly shorter at 1.92 and 1.91 Å in **aRC** and **bRC** respectively (Figure 1). This shortening is likely assisted by Asp383 which is still hydrogen bonded via its carboxylate to the Thr-OH group.

In addition, in both **aRC** and **bRC** there is a hydrogen bonding bridge network between the imidazole of His309 and the threonyl's carbonyl oxygen (Figure 1). However, due to their different protonation states of the His309 residue, they exhibit several notable differences. Specifically, in **aRC** the protonated imidazole of His309 acts as a hydrogen bond donor to the 2'O of the ^{Thr}tRNA moiety. Due to the fact that this interaction involves a charged moiety, the resulting $\text{His309NH}^+\cdots\text{O2'}_{\text{A76}}$ interaction is quite short and strong with a length of just 1.53 Å and a BCP value (0.070; Table 1), that is well above the range of typical H-bonds (see above).^{53,54} The A762'OH group itself then acts as a H-bond donor to the active site H_2O with an $r(\text{A762'OH}\cdots\text{OH}_2)$ length of 1.66 Å. Again this is also a quite short and strong hydrogen bond as is also reflected in the relatively high value of ρ (0.051) at its BCP. As noted above, the H_2O moiety also forms a moderately strong interaction with the threonyl's $\text{C}_{\text{carb}}=\text{O}$ oxygen.

In contrast, in **bRC** the now unprotonated imidazole N centre of His309 (N_{His309}) acts as a hydrogen bond *acceptor* towards the A762'OH group (Figure 1). It is noted that this is now a neutral interaction, the resulting $\text{His309N}\cdots\text{HO2'}_{\text{A76}}$ distance (1.93 Å) is 0.40 Å longer than the corresponding interaction in **aRC**. Its now more typical H-bond length is also illustrated by the fact that at its BCP (^bB1) the values of ρ (0.031) and ρ_2 (0.074) now lie *within* the standard range of such interactions (Table 1). As a consequence, the 2'-oxygen now forms a longer though still moderately strong hydrogen bond to the active site H_2O with a $2'\text{O}\cdots\text{H}_2\text{O}$ length of 1.90 Å ($\rho=0.028$; Table 1). As noted above, the H_2O in **bRC** is also simultaneously hydrogen bonded to the threonyl's $\text{C}_{\text{carb}}=\text{O}$ oxygen. In addition, however, it also forms a weak hydrogen bond with the threonyl's $-\text{NH}_2$ group; $r(\text{H}_2\text{O}\cdots\text{HN}_{\text{Thr}})=2.44$ Å. This is also reflected in its ρ (0.011) and ρ_2 (0.045) values which lie towards the lower end of the ranges usually observed for hydrogen bonds.^{53,54} Thus, in **bRC** the His309 residue is involved in a hydrogen bond bridge with both the threonyl's $\text{C}_{\text{carb}}=\text{O}$ and $-\text{NH}_2$ groups via A762'OH and the active site H_2O (Figure 1).

It is noted that we also considered larger active site models that also included Tyr462, thought to also interact with A762'OH , and Gln484 which was found to hydrogen bond via its R-group oxygen with the threonyl's $-\text{NH}_2$ (not shown). These models were found to give overall structures in close agreement with that of **aRC** and **bRC** above.

In our previous MD study⁴⁰ on the fully bound active site of ThrRS in which His309 was either neutral or protonated, the structures of both resulting complexes were observed to be in reasonable agreement with experiment. Indeed, as detailed above, the major difference is the hydrogen bond network centered on the A762'OH group. In addition, at the level of theory used in the present study the R-group of His309 is calculated to have similar proton affinities whether it is within the active site (1195.0 kJ mol⁻¹) or in aqueous solution (1192.9 kJ mol⁻¹). Thus, for completeness and to obtain greater insights we have considered possible pathways in which His309 is protonated (Section 3.2) or neutral (Section 3.3).

3.2. The protonated His309-assisted mechanism

The potential energy surface (PES) obtained for the catalytic mechanism of ThrRS when His309 is protonated is shown schematically in Figure 2. The optimized structure of each intermediate and transition structure (TS) along the pathway is provided in Figure 3. As can be seen, for the chemical model used herein the overall mechanism can be thought of as occurring in three stages: (i) formation and (ii) rearrangement of a tetrahedral intermediate complex and (iii) formation of the product complex.

Formation of the tetrahedral intermediate complex

This initial stage of the overall mechanism occurs in two steps. First, the His309-H⁺ residue essentially transfers its proton via the _{A76}2'OH and active site H₂O onto the threonyl's C=O oxygen. This process occurs via transition structure **^aTS1** (obtained via detailed scans) with a moderately low barrier of 71.6 kJ mol⁻¹ to give the intermediate complex **^aIC1** lying only 0.2 kJ mol⁻¹ lower in energy at 71.4 kJ mol⁻¹. These almost equivalent energies are reflected in their optimized structures with the largest difference in the bond lengths for **^aTS1** and **^aIC1** shown in Figure 3 being just 0.02 Å.

In **^aIC1** the His309 residue is neutral and forms a strong hydrogen bond chain to the threonyl's now protonated C=OH⁺ group via the _{A76}2'OH and H₂O moieties. This protonation causes a slight increase in the charge on threonyl's C_{carb} centre from 0.63 (**^aRC**) to 0.68, thus enhancing its electrophilicity. It is noted that while the C=OH⁺ bond has lengthened from 1.23 (**^aRC**) to 1.27 Å, the C_{carb}—OP bond has shortened by almost the same amount from 1.32 (**^aRC**) to 1.28 Å due to the now greater positive charge on C_{carb}.

His309-assisted mechanism of ThrRS. For clarity, only some residues are shown in ball and stick format. Color key: C (gray); O (red); N (blue); P (orange); S (yellow), Zn (lavender) and H (white).

Indeed, this step is followed by nucleophilic attack of the _{A76}3'O at the threonyl's C_{carb} centre. This proceeds via **^aTS2** at a cost of 70.0 kJ mol⁻¹ with respect to **^aIC1**. More importantly, however, the overall barrier of this step is 141.4 kJ mol⁻¹ with respect to the initial bound active site complex **^aRC**; 155.0 kJ mol⁻¹ with respect to the pre-reactive complex **^aPRC**! As seen in Figure 2, this is the rate-limiting step in the overall protonated His309-assisted mechanism. Importantly, however, it is significantly higher than that generally considered to be the upper thermodynamic limit for an enzyme catalysed reaction: ~ 125 kJ mol⁻¹.³⁹ Furthermore, the formation of the threonyl-tRNA C_{carb}—O3'_{A76} bond occurs with concomitant transfer (Figure 3: **^aTS2**) of the _{A76}3'OH proton to the threonyl's -NH₂ group. The resulting tetrahedral intermediate complex **^aIC2** lies 86.0 kJ mol⁻¹ higher in energy than **^aRC**. In **^aIC2** the C_{carb}—O3'_{A76} crosslink formed has a length (1.43 Å) that is typical for C—O single bonds while both the C_{carb}—OH and C_{carb}—OP bonds have concomitantly lengthened significantly by 0.12 and 0.10 Å to 1.39 and 1.38 Å, respectively (Table S1). Moreover, the threonyl's now -NH₃⁺ group hydrogen bonds via the H₂O to the _{A76}2'OH, which itself still interacts with the His309 residue (Figure 3).

As a note here, we did consider the direct attack of the 3'O to C_{carb} prior to the proton transfer to the carbonyl oxygen in **^aRC**. The barrier for this process was found to be 174.0 kJ mol⁻¹ with respect to **^aRC** (not shown). Thus while the initial proton transfer from his309NH⁺ has lowered the barrier by ~ 30 kJ mol⁻¹, it remains too high to be enzymatically feasible.

Rearrangement of the tetrahedral intermediate complex

Prior to cleavage of the PO—C_{carb} bond (completing the transfer of the threonyl moiety), within the present computational model, the tetrahedral intermediate must undergo a two-step rearrangement. This rearrangement guarantees the regeneration of His309-H⁺ within the mechanism. It begins with rotation about the C_{carb}—OH bond that no longer hydrogen bonds with the H₂O but instead now interacts with a non-bridging phosphate oxygen (Figure 2). This step proceeds via **^aTS3** (obtained via detailed scans) at a low cost of approximately 8.6 kJ mol⁻¹. The alternate tetrahedral complex formed (**^aIC3**) lies markedly lower in energy than **^aIC2** by 40.6 kJ mol⁻¹; just 45.4 kJ mol⁻¹ higher than **^aRC**. The formation of a short C_{carb}OH...OP hydrogen bond (1.66 Å) results in minor changes to the C_{carb}—OP and

$C_{\text{carb}}\text{—OH}$ bonds of +0.02 to 1.40 Å and −0.01 to 1.38 Å, respectively. In addition, however, the threonyl's —NH_3^+ group now directly interacts with the $A_{76}2'\text{OH}$ group; $r(\text{NH}\cdots\text{O}2'_{A76}) = 1.93$ Å (Figure 3).

Subsequently, the —NH_3^+ moiety transfers a proton via $A_{76}2'\text{OH}$ to the imidazole of His309, thus regenerating His309- H^+ , i.e., protonated His309. This step proceeds via **^aTS4** at a cost of 48.8 kJ mol^{−1} with respect to **^aIC3** to give a higher energy tetrahedral intermediate **^aIC4** having a relative energy of 71.0 kJ mol^{−1} with respect to **^aRC**. In **^aIC4** the $C_{\text{carb}}\text{OH}\cdots\text{OP}$ hydrogen bond has shortened slightly to 1.64 Å, resulting in a minor increase (0.01 Å) in the $C_{\text{carb}}\text{—OP}$ bond to 1.41 Å, while the His309 residue is now once again protonated (Figure 3).

Formation of the product complex

The last step in the aminoacyl transfer process is cleavage of the $C_{\text{carb}}\text{—OP}$ bond to give the final corresponding product complex (**^aPC**). This step occurs via the six-membered ring transition structure **^aTS5** at a quite low cost of only 12.2 kJ mol^{−1} with respect to **^aIC4**. The final product-bound active site complex **^aPC** lies slightly higher in energy than the initial substrate-bound active site complex **^aRC** by 7.0 kJ mol^{−1}. In **^aPC** the cleaved $C_{\text{carb}}\cdots\text{OP}$ distance is now 3.29 Å while both the $C_{\text{carb}}=\text{O}$ and $C_{\text{carb}}\text{—O}3'_{A76}$ bonds have both shortened markedly to 1.22 and 1.34 Å, respectively.

Thus, with His309 initially protonated, the preferred pathway occurs via an acid catalysed esterification.⁵⁵ His309- H^+ acts as the initial acid protonating the substrates carbonyl oxygen while the $A_{76}3'\text{OH}$ loses its proton via donation to the substrates —NH_2 group. However, the barrier for the rate-limiting step (141.4 kJ mol^{−1}) for the formation of the tetrahedral intermediate **^aIC2**, is above the generally held thermodynamic 'upper-limit' for *in vivo* enzymatic processes (~125 kJ mol^{−1}).³⁹

3.3. The neutral His309-assisted mechanism

The PES obtained for the catalytic mechanism of ThrRS with a neutral His309 is shown schematically in Figure 4 while the optimized structure of each intermediate and TS involved, with selected bond lengths is given in Figure 5.

Formation of a tetrahedral intermediate complex—In contrast to that observed in the protonated His309-assisted mechanism, the tetrahedral intermediate is formed from the reactive complex (**^bRC**) in a single step. This occurs via **^bTS1** at a cost of 106.8 kJ mol^{−1} or 114.3 kJ mol^{−1} with respect to the pre-reactive complex **^bPRC**. It is noted that this barrier is close to that previously obtained for HisRS where the analogous nucleophilic attack of 3'OH to give a tetrahedral intermediate was calculated to be approximately 109.2 kJ mol^{−1}.⁵ More importantly, in contrast to that observed in the protonated His309 assisted mechanism, this barrier is *lower* than that generally held to be the upper thermodynamic enzymatic limit.^{39,56,57} It should be noted that as observed in the analogous TS of the 'protonated, His309' pathway, **^aTS2** (cf. Figure 3), in **^bTS1** the $A_{76}3'\text{OH}$ oxygen nucleophilically attacks at the substrates' C_{carb} centre with concomitant transfer of the 3'OH proton to the threonyl substrates' —NH_2 group. Now, however, the 3'OH proton is almost fully transferred onto the Thr- NH_2 group as indicated by the $3'\text{O}\cdots\text{H}$ and $3'\text{OH}\cdots\text{N}_{\text{Thr}}$ distances of 1.61 and 1.08 Å respectively (Figure 5), while the forming $C_{\text{carb}}\cdots\text{O}3'$ bond (1.96 Å) is now much longer (cf. Figure 3).

The resulting oxyanion tetrahedral intermediate **^bIC1** lies 85.6 kJ mol^{−1} higher in energy relative to **^bRC**. As can be seen in Figure 5 the $C_{\text{carb}}\text{—O}3'_{A76}$ bond has now been formed with a length of 1.51 Å while the former $C_{\text{carb}}=\text{O}$ and $C_{\text{carb}}\text{—OP}$ bonds have significantly

lengthened to 1.30 and 1.43 Å respectively (Table S1), and the threonyl's amino group is now protonated (i.e., -NH_3^+). The $\text{C}_{\text{carb}}\text{---O}^-$ oxyanion is stabilized in part via a strong hydrogen bond (1.47 Å) with the H_2O which is itself hydrogen bonded to both the -NH_3^+ (1.87 Å) and $\text{A}_{76}2'\text{OH}$ (2.40 Å) groups. It is noted that the -NH_3^+ also forms weak hydrogen bonds with both the $\text{A}_{76}3'\text{O}$ and $\text{A}_{76}2'\text{O}$ with distances of 2.17 and 2.08 Å, respectively.

We did also examine stepwise and concerted pathways in which His309 may directly act as a base by accepting a proton from $\text{A}_{76}2'\text{OH}$ as previously proposed.²¹ However, within the present computational model as well as the larger model that also included the residues Tyr462 and Gln484, no stable intermediate in which threonyl's amino group remains neutral while His309 is fully or partially protonated was obtained. That is, **^bIC1**-type intermediates were the only stable oxyanionic intermediate obtained.

Formation of the 'neutral' product complex—Similar to that observed in the 'protonated His309'-assisted mechanism, within the present computational model, the tetrahedral intermediate **^bIC1** must then undergo a two-step rearrangement prior to formation of the final product complex. The first step occurs with a proton transfer from the -NH_3^+ moiety via the H_2O to the oxyanion $\text{C}_{\text{carb}}\text{---O}^-$ centre. This process is essentially barrierless (**^bTS2**) to give the alternate tetrahedral intermediate **^bIC2** lying 3.5 kJ mol^{-1} lower in energy than **^bIC1** (Figure 4). In **^bIC2** the now 'neutralized' $\text{C}_{\text{carb}}\text{---OH}$ bond has lengthened markedly by 0.08 Å to 1.38 Å while both the $\text{C}_{\text{carb}}\text{---OP}$ and $\text{C}_{\text{carb}}\text{---O3a}_{76}$ bonds have shortened to 1.39 and 1.44 Å, respectively (Table S1). It is noted that in **^bIC2** the active site H_2O forms a hydrogen bond bridge between the -NH_2 (1.77 Å), $\text{A}_{76}2'\text{OH}$ (1.96 Å) and $\text{C}_{\text{carb}}\text{OH}$ (1.83 Å) as shown in Figure 5.

The second step, again is similar to that observed in the 'protonated His309'-assisted mechanism; rotation about the $\text{C}_{\text{carb}}\text{---OH}$ bond. However, this rotation proceeds via **^bTS3** with an approximately 16 kJ mol^{-1} lower barrier of only 32.5 kJ mol^{-1} ; 114.6 kJ mol^{-1} with respect to **^bRC** (Figure 4). Furthermore, the resulting alternate tetrahedral conformer **^bIC3** formed lies just 5.2 kJ mol^{-1} higher in energy than **^bIC2** (cf. Figure 2). In **^bIC3** the $\text{C}_{\text{carb}}\text{OH}$ group now forms a moderately short hydrogen bond (1.77 Å) to a non-bridging phosphate oxygen (Figure 5). Importantly, this distance is longer than observed in **^aIC4** (cf. Figure 3) causing a slight lengthening in the $\text{C}_{\text{carb}}\text{---OP}$ bond to 1.43 Å (Table S1).

With the rotation of the hydroxyl group, as observed in the 'protonated His309'-assisted mechanism, the final step is then the concomitant transfer of the proton from the $\text{C}_{\text{carb}}\text{---OH}$ to the non-bridging phosphate oxygen and cleavage of the $\text{PO}\text{---C}$ bond. This occurs via the six-membered ring transition structure **^bTS4** at a cost of only 27.0 kJ mol^{-1} , 114.3 kJ mol^{-1} with respect to the initial substrate-bound active site complex **^bRC**. In contrast, however, the resulting product-bound active site complex **^bPC** is calculated to lie lower in energy than the initial substrate-bound active site complex **^bRC** by 24.4 kJ mol^{-1} (Figure 5). In **^bPC** the $\text{C}_{\text{carb}}\cdots\text{OP}$ distance is now 5.73 Å while the $\text{C}_{\text{carb}}\text{---O3'}_{\text{A}_{76}}$ and $\text{C}_{\text{carb}}\text{=O}$ bonds have shortened significantly to 1.35 and 1.22 Å, respectively. The threonyl has been transferred onto the $\text{tRNA}_{\text{A}_{76}\text{atits3'}}$ -oxygen.

In the above 'neutral His309' assisted mechanism, the highest overall mechanism barriers are the rotation about the $\text{C}_{\text{carb}}\text{---OH}$ bond and proton transfer from $\text{C}_{\text{carb}}\text{---OH}$ to a non-bridging phosphate oxygen. With regards to **^bRC**, as noted above, these barriers are 114.6 and 114.3 kJ mol^{-1} while with regards to the pre-reactive complex **^bPRC** these barriers are 122.1 and 121.8 kJ mol^{-1} respectively. These barriers are still enzymatically feasible, though they are near the generally-held upper enzyme-catalysed thermodynamic limit.³⁹ However, a possible alternate pathway is direct cleavage of the $\text{C}_{\text{carb}}\text{---OP}$ bond in the

tetrahedral intermediate **IC1** to give a product complex consisting of bound AMP (modeled as methylphosphate) and aminoacylated-^tRNA^A76 (modeled by dextro-ribose threonyl); i.e., a two-step process. However, in order to model this route, a larger chemical model was required that also included Lys465 (modeled as an unconstrained protonated methylamine) in order to help stabilize the charge on the phosphate.

The resulting PES obtained is shown in Figure 6. It should be noted that the optimized structures of all stationary points along the pathway were very similar to their corresponding counterparts in Figures 4 and 5, the main differences being relatively minor changes in the non-bridging P—O bond lengths and that in the product complex where the threonyl amino group remains protonated. As can be seen, inclusion of the lysyl has little effect on the barrier for formation of the tetrahedral intermediate **^bIC1'** which proceeds via **^bTS1'** with an almost negligibly lower barrier of 105.1 kJ mol⁻¹ (cf. Figure 4). Indeed, in mutagenesis studies a Lys465Ala (K465A) substitution had only minor effects on the reaction kinetics.²¹ In this step the 3'OH proton is again transferred directly to the Thr-NH₂ group. Furthermore, the product complex **^bPC'** still lies markedly lower in energy than the initial reactive complex **^bRC'** by 15.8 kJ mol⁻¹. Importantly, however, the tetrahedral complex **^bIC1'** lies 52.8 kJ mol⁻¹ higher in energy than **^bRC'** and cleavage of the C_{carb}—OP bond, formal transfer of the aminoacyl moiety, now occurs directly via **^bTS2'** without a barrier to give the product **^bPC'**. Thus, the rate-limiting step is formation of the tetrahedral intermediate as observed in related computational studies.⁵

4. Conclusions

In this current investigation, density functional theory-based methods in combination with large chemical models have been applied to the second half-reaction catalyzed by Thr-tRNA synthetase. Specifically, transfer of the threonyl aminoacyl moiety from Thr-AMP onto the ribose 3'O of the A76 residue of the cognate ^{Thr}tRNA. Furthermore, we have considered both cases in which an active site histidyl residue (His309) is either protonated or neutral.

For each resulting initial substrate bound-active site complex two alternate binding modes for the threonyl moiety were obtained. In the lowest energy complexes (**^aPRC** and **^bPRC**) the threonyl moiety was bidentate-coordinated to the Zn(II) ion via its R-group hydroxyl oxygen and α -amino nitrogen (N_{Thr}) centre. In the corresponding alternate reactive complexes **^aRC** and **^bRC** the Zn(II)···N_{Thr} bond is cleaved and the threonyl is now only monodentately ligated to the Zn(II) centre via its R-group hydroxyl oxygen. Furthermore, they lie only slightly higher in energy than **^aPRC** and **^bPRC** by 13.6 and 7.5 kJ mol⁻¹ respectively. Thus, the Zn(II)···N_{Thr} bond appears to be reasonably labile, particularly in the neutral His309 system.

In the aminoacyl transfer pathway obtained for when the His309 is initially protonated, the rate-limiting step is formation of the tetrahedral intermediate. That is, the step in which the 3'OH oxygen nucleophilically attacks the threonyl's carbonyl carbon centre. However, the barrier is 141.4 (155.0) kJ mol⁻¹ with respect to **^aRC** (**^aPRC**). Consequently, it is not enzymatically feasible. Notably, in this step the 3'OH proton is not transferred to the His309 residue, but instead to the Thr-NH₂ group.

Two models were considered in which the His309 residue was initially neutral, the difference being whether Lys465 was included, simply in order to help counterbalance the negative charge on the phosphate in the model. For both models the rate-limiting step was found to be below the enzymatic limit of 125 kJ mol⁻¹. In particular, for the model containing Lys465 the rate limiting step was formation of the tetrahedral intermediate. The barrier being approximately 105 kJ mol⁻¹ with respect to **^bRC**. For the model which did not

include Lys465, the rate limiting step was rotation about the C_{carb}—OH bond. The barrier being approximately 114.6 kJ mol⁻¹ with respect to **bRC**; 122.1 kJ mol⁻¹ with respect to **bPRC**. However, while the rate limiting steps differed for the two models the barriers for formation of the tetrahedral intermediate were very similar and differed by only 1.7 kJ mol⁻¹. More importantly, however, is that for both models, the 3'OH proton is not transferred to the His309 residue. Rather, during formation of the tetrahedral intermediate it directly transfers from the 3'OH onto the threonyl moieties -NH₂ group.

Thus, the current results suggest that as observed for other aminoacyl-tRNA synthetases,^{16,17,20} ThrRS utilizes a substrate-assisted catalysis mechanism in the aminoacyl transfer for ThrRS. However, it is not the threonyl-AMP substrate's phosphate group that acts as the base but rather its -NH₂ group. The presence of the Zn(II) ion and lability of the Zn(II) ⋯N_{Thr} bond appears to allow the -NH₂ to act as the mechanistic base.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Optimized structures with selected bond distances (Angstroms) of the fully bound active site pre-reactive and reactive complexes in which His309 is protonated (**aPRC** and **aRC** respectively) or neutral (**bPRC** and **bRC** respectively).

Figure 2.
Calculated PES for the protonated His309-assisted mechanism of ThrRS.

Figure 3.
Optimized structures with selected bond lengths (Angstroms) of the intermediates (IC), transition structures (TS) and product complex (PC) of the protonated

Figure 4.
Calculated PES for the neutral His309-assisted mechanism of ThrRS.

Figure 5. Optimized structures with selected bond lengths (Angstroms) of the intermediates (**IC**), transition structures (**TS**) and product complex (**PC**) of the neutral His309-assisted mechanism of ThrRS. For clarity, only key residues are shown in ball and stick format. Color key: C (gray); O (red); N (blue); P (orange); S (yellow), Zn (lavender) and H (white).

Figure 6. Calculated PES for the neutral His309-assisted mechanism of ThrRS in which the lysyl residue (Lys465) is included in the chemical model (see text).

Scheme 1.

General catalytic mechanism of aaRS's in which the substrates *pro-S* non-bridging oxygen acts as the mechanistic base.¹⁶

Scheme 2.
Proposed mechanism of ThrRS.²¹

Scheme 3.

Schematic illustration of the two ThrRS active site models used in this study in which His309 (a) is neutral and acts as a base and (b) is protonated and acts as an acid.

Table 1

Values of the Electron Density (ρ) and its Laplacian ($\nabla^2\rho$) (a.u.) at BCPs of Selected Hydrogen Bonds Within the Fully Bound Reactive Complexes **^aRC** and **^bRC**.^a

	BCP	ρ	$\nabla^2\rho$
^aRC			
^a B1	A76 2'O... ⁺ HN _{His309}	0.070	0.154
^a B2	A76 3'OH...N _{Thr}	0.034	0.076
^a B3	A76 2'OH...OH ₂	0.051	0.140
^a B4	OH ₂ ...O=C _{carb}	0.026	0.073
^bRC			
^b B1	A76 2'OH...N _{His309}	0.031	0.074
^b B2	A76 3'OH...N _{Thr}	0.033	0.076
^b B3	OH ₂ ...A76 2'O	0.028	0.083
^b B4	OH ₂ ...O=C _{carb}	0.032	0.097

^a Small red points indicate BCP's. Color key: C (gray); O (red); N (blue); P (maroon); S, yellow); H (grey) and Zn (white).