**ABSTRACT:** Human PAICS is a bifunctional enzyme that is involved in the *de novo* purine biosynthesis, catalyzing the conversion of aminoimidazole ribonucleotide (AIR) into N-succinylcarboxamide-S-aminoimidazole ribonucleotide (SAICAR). It comprises two distinct active sites, AIR carboxylase (AIRc) where the AIR is initially converted to carboxyaminoimidazole ribonucleotide (CAIR) by reaction with CO, and SAICAR synthetase (SAICARs) in which CAIR then reacts with an aspartate to form SAICAR, in an ATP-dependent reaction. Human PAICS is a promising target for the treatment of various types of cancer, and it is therefore of high interest to develop a detailed understanding of its reaction mechanism. In the present work, density functional theory calculations are employed to investigate the PAICS reaction mechanism. Starting from the available crystal structures, two large models of the AIRc and SAICARs active sites are built and different mechanistic proposals for the carboxylation and phosphorylation–condensation mechanisms are examined. For the carboxylation reaction, it is demonstrated that it takes place in a two-step mechanism, involving a C–C bond formation followed by a deprotonation of the formed tetrahedral intermediate (known as isoCAIR) assisted by an active site histidine residue. For the phosphorylation–condensation reaction, it is shown that the phosphorylation of CAIR takes place before the condensation reaction with the aspartate. It is further demonstrated that the three active site magnesium ions are involved in binding the substrates and stabilizing the transition states and intermediates of the reaction. The calculated barriers are in good agreement with available experimental data.

1. **INTRODUCTION**

The *de novo* purine biosynthesis pathway comprises a series of reaction steps converting phosphoribosyl pyrophosphate into the final product, inosine monophosphate, which represents a key building block in nucleotide metabolism. The pathway is ubiquitous in all domains of life. Eukaryotic *de novo* purine biosynthesis pathway includes 10 enzymatic reactions, and in humans, reaction steps 6 and 7 are catalyzed by the bifunctional enzyme PAICS (phosphoribosylaminomimidazole carboxylase and phosphoribosylaminomimidazolesuccinocarboxamide synthetase).

Cancer cells strongly depend on the *de novo* purine biosynthesis pathway, in contrast to healthy cells, which preferentially produce purines through the nucleotide salvage pathway. Therefore, all enzymes that participate in the *de novo* purine biosynthesis pathway can be considered potential targets for anticancer therapy. Indeed, PAICS is overexpressed in several types of cancer and has been shown to play an important role in cancer cell proliferation and invasion, correlating therefore with poor prognosis. Accordingly, PAICS has been explored as a potential cancer therapy target. Its knockdown resulted in reduced tumor growth in a mouse model of prostate cancer, and shRNA-mediated PAICS depletion reduced tumor growth in mouse models of lung, pancreas, breast, colorectal, and gastric cancer. PAICS has also been suggested as a target for treating neuroblastoma. A recent study has shown that pharmacologic inhibition of PAICS by a recently developed inhibitor had an antileukemia effect both in *vitro* and *in vivo* in a mouse xenograft model of acute myeloid leukemia, thus identifying PAICS as a chemotherapy target.

Substrate/product-based inhibitors of PAICS homologues have been synthesized in the past as tools in enzymatic analysis and recent efforts have been made to develop inhibitors of prokaryotic PAICS homologues to combat antibiotic resistance. In animals, the bifunctional enzyme PAICS catalyzes sequentially the carboxylation of aminoimidazole ribonucleotide (AIR) to carboxyaminoimidazole ribonucleotide (CAIR) and, in the following reaction, the condensation of CAIR and L-aspartate (Asp) to N-succinylcarboxamide-S-aminoimidazole ribonucleotide (SAICAR). The latter reaction takes place in an

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The AIR carboxylase (AIRc) and SAICAR synthetase (SAICARs) activities are combined in one polypeptide chain (see Figure 1). While the biosynthesis of SAICAR is universal across the tree of life, CAIR is synthesized in two different ways. In animals, the bifunctional PAICS contains a type II PurE domain (AIRc) that catalyzes the biosynthesis of CAIR in one step, as a direct carboxylation of AIR by CO$_2$ at C$^4$ (see Figure 1). This is in contrast to the catalytic strategy adopted by plants, yeast, and most prokaryotes, which synthesize CAIR in two steps using two enzyme activities. In the first reaction, PurK catalyzes the carboxylation of AIR at N$^5$ with bicarbonate, in the presence of ATP. The product, N$^5$-CAIR, is then converted to CAIR by an N$^5$-CAIR mutase (type I PurE, a homolog of PurE-II).

The structural characterization of prokaryotic PurE-I and PurE-II, prokaryotic and yeast SAICARs (PurC) enzymes, as well as bifunctional PAICS enzymes, including their complexes with natural ligands (substrates and products) has led to a number of mechanistic proposals for PAICS. More specifically, two alternative proposals have been put forward for the carboxylation of AIR, common to both the PurE-II reaction and the second PurE-I half-reaction, and three mechanisms have been proposed for SAICARs (see the Results and Discussion section below).

A detailed understanding of the catalytic mechanism of eukaryotic bifunctional PAICS and the molecular structure of intermediates and transition states is of crucial importance for emerging cancer therapies targeting human PAICS, because it can aid the rational design of novel PAICS inhibitors. For this reason, we report here a detailed computational investigation of the reaction mechanism of human PAICS, adopting the quantum chemical cluster approach, a methodology that has been successfully applied to study a vast number of enzymatic systems. We focus on the three chemical reactions involved in the conversion of AIR to SAICAR, namely, carboxylation in AIRc and phosphorylation and condensation in SAICARs. We use two large active site models, one for the AIRc and one for the SAICARs catalytic pocket. The results of the investigation are in good agreement with available experimental observations. They shed light on the source of the catalytic power of the bifunctional enzyme and help to rule out several suggestions regarding the chemical steps toward SAICAR.

The current computational study can thus contribute to the rational design of potent and selective PAICS inhibitors as novel anticancer therapeutics.

2. COMPUTATIONAL METHODS

2.1. Active Site Models. Two active site models were designed from the recently solved crystal structures of PAICS from Homo sapiens, in complex with CAIR (PDB 6YB8) and with SAICAR and AMP-PNP, an analogue of ATP (PDB 6YB9).

In the AIRc active site model, the CAIR product from the crystal structure was manually replaced by the AIR and CO$_2$ substrates. The model consists of the amino acids composing the active site and interacting with the substrates: Met272, Gly273, Ser274, Thr275, Ser276, Asp277, Val278, Ala329, Gly330, Arg331, Ser332, Asn333, Gly334, Leu335, Ser336, Ala302, His303, and Lys304. One crystallographic water, bridging Ser332 and His303, was also explicitly included. The final size of the model is 269 atoms and the total charge is 0.

In the SAICARs active site model, the CAIR and Asp substrates were obtained by splitting the SAICAR product from the crystal structure, while AMP-PNP was changed into ATP. The amino acids belonging to the ATP binding site (Gly16, Lys17, Thr18, and Lys19) and SACAIR binding site (Thr40, Ala41, Gly42, Asn43, Gln97, Val99, Lys131, Asp133, Asp137, Phe129, Met190, Lys191, Gln193, Asp207, Asp212, Ser213, Trp214, Arg215, Lys228, Arg232) were included. In addition, three hexa-coordinated Mg ions (Mg$_A$, Mg$_B$, and Mg$_C$; see Figure 2) and seven water molecules coordinating to the metals were included in the model, resulting in a model size of 369 atoms and an overall charge of −1. Mg$_A$ and Mg$_B$ were present in the crystal structure, while Mg$_B$ was added manually in the model. Although Mg$_B$ is absent in the electron density map of the H. sapiens PAICS crystal structure, its existence is supported by enzyme kinetics experiments indicating that a higher number of Mg ions are required for catalysis than those required for the saturation of ATP. This thus
suggests that the enzyme carries out the catalytic activity in the presence of three cations. In addition, the crystal structure of SAICARs from *E. coli* in complex with ADP and CAIR contains three ions, providing further evidence for the presence of Mg in PAICS. Accordingly, the position of Mg in the active site model was identified by the superposition of the *H. sapiens* crystal structure with the one of SAICARs:ADP:CAIR from *E. coli*, which contains all three metal ions (see Figure S1).13j

The amino acids, cofactors, and substrates were truncated, as shown in Figure 2. Hydrogen atoms were added manually to saturate the carbon atoms. The phosphate group of the AIR substrate was modeled in the dianionic state. However, during the geometry optimizations, a proton moved over spontaneously from the positively charged Lys304 to one of the oxygens. The Asp substrate in the phosphorylation-condensation reaction was modeled in the dianionic state, i.e., with the two carboxylates in the deprotonated form (−COO−) and a neutral amino group (−NH2). This choice is based on visual inspection of the active site showing that the two carboxylate moieties can form salt bridges to several positively charged groups (Arg232, Lys228, and Lys191), while the amino group can accept a hydrogen bond from the CAIR substrate and in the neutral form can act as a nucleophile in the reaction (see below).

A number of carbon atoms, typically where the truncation was made (labeled with “*” in Figure 2), were kept fixed during the geometry optimizations to avoid large artificial movements as compared to the crystal structure. This procedure leads to a number of imaginary frequencies (<50 cm−1) that can be ignored since they do not affect the relative energies of the optimized structures.16a

2.2. Computational Details. All calculations were carried out using the B3LYP-D3(BJ) functional17 as implemented in Gaussian16 C.01.18 The geometry optimizations were performed employing the LANL2DZ pseudopotential19 for Mg and the 6−31G(d,p) basis set for other atoms. At the same level of theory, the effect of protein surrounding was calculated using single-point energy calculations with the solvation model based on density (SMD) solvation method (ε = 4),20 and frequency calculations were performed to obtain zero-point energy (ZPE). The entropy contribution from the inclusion of a small gas molecule was estimated to be equal to the translational entropy of
the free molecule. The calculated entropy for CO$_2$ is 11.1 kcal/mol at room temperature, which is added to the energy of the formation of the enzyme−CO$_2$ adduct, a procedure used previously in other studies. To obtain more accurate electronic energies, single-point energy calculations were performed using the LANL2DZ basis set for Mg and the larger 6−311+G(2d,2p) basis set for other atoms. The energies presented in the paper are the larger basis set energies including solvent and ZPE corrections.

3. RESULTS AND DISCUSSION

The complete catalytic mechanism of the human PAICS proposed here is characterized by three different reactions, taking place consecutively in the AIRc and SAICARs subunits of the enzyme (Scheme 1). The AIR substrate is first carboxylated in the AIRc active site by reaction with CO$_2$, thus generating CAIR. Subsequently, CAIR is phosphorylated by ATP in the SAICARs active site, followed by a reaction with Asp to yield the SAICAR product. The mechanistic details of each reaction, as obtained by the current calculations, are presented in the following sections.

3.1. Carboxylation in the AIRc Active Site. The energy profile of the carboxylation step is depicted in Figure 3. In the optimized enzyme−substrate complex (E:AIR), the aminoimidazole ring of the substrate is involved in hydrogen bonds with the side chain of Ser301 and the backbone carbonyl of Gly330, while the hydroxyl groups of the ribose ring interact with the carboxylate moiety of Asp277 (see Figure 4). The phosphate group of the substrate is surrounded by the side chain of Lys304 and by the hydroxyl groups of Ser274 and Ser276, thus maintaining a similar conformation as in the crystal structure (see superposition in Figure S2). Comparison of the conformations reveals that the guanidinium group of Arg331 shifts slightly with respect to its position in the crystal structure, favoring the interaction with the phosphate group of AIR. We also note that since the AIR substrate lacks the carboxylate moiety as compared to the CAIR product in the crystal structure, the positions of Asn333, Gly334, and Leu335 in the E:AIR complex are somewhat shifted compared to that structure (Figure S2).

Next, CO$_2$ binds to the AIRc active site. The calculations show that it can bind in different positions relative to the AIR substrate, i.e., either in the pocket occupied by the carboxylate moiety of the CAIR product in the crystal structure (the complex is called E:AIR:CO$_2$, Figure 4) or on the other side of the aminoimidazole ring toward the solvent-exposed area (called E:AIR:CO$_2'$, see Figure S3). E:AIR:CO$_2'$ is energetically more favorable than E:AIR:CO$_2$ (+5.8 vs +14.9 kcal/mol, respectively, compared to the E:AIR complex). However, although E:AIR:CO$_2$ is higher in energy, it is the only productive binding mode, as C−C bond formation starting from E:AIR:CO$_2'$ is associated with prohibitively high energies (see Figure S3).

In E:AIR:CO$_2$, the CO$_2$ binding site is located at the bottom of the active site pocket, below the bound AIR. The oxygen atoms of CO$_2$ engage in interactions with the amide groups of Ala302 and Gly334 (Figure 4). The presence of CO$_2$ causes some shift in the positions of the amino acids of the binding site of the gas molecule, without substantially altering...
the hydrogen-bonding interactions of the AIR substrate, as can be seen from the superposition of the optimized E:AIR and E:AIR:CO₂ structures provided in Figure S4. Furthermore, the position of CO₂ in its binding site in the E:AIR:CO₂ model is in good agreement with the structure of Acetobacter aceti PurE-II in complex with AIR and CO₂ (PDB 5CLJ).

From E:AIR:CO₂, the carbon dioxide is next attacked by the C=C double bond of AIR with a barrier (TS₁) of 3.0 kcal/mol relative to E:AIR:CO₂ (i.e., 17.9 kcal/mol relative to E:AIR), generating INT₁, which lies at +10.1 kcal/mol. Upon C−C bond formation, the aminoimidazole ring of the substrate in INT₁ is tetrahedral at the C₄ position and represents thus the nonaromatic form of CAIR (named isoCAIR). This species has been suggested as an intermediate in the second half-reaction of PurE-I₁₃ and has also been implicated in the nonenzymatic decarboxylation of CAIR.²² In INT₁, two hydrogen-bonding interactions between the formed carboxylate group and the amide NH groups of the peptide bonds of Ala302 and Gly334 help to stabilize the intermediate (see structures in Figure S5).

In the next step of the reaction, a proton transfer takes place from the C₄ of the substrate to His303, resulting in the enzyme—product complex E:CAIR. The calculated energy barrier for this step (TS₂) is +18.0 kcal/mol and E:CAIR is 10.1 kcal/mol higher than E:AIR. The conformations of the active site residues in E:CAIR are in agreement with the available X-ray structure of human PAICS, as can be seen from the superposition of the two structures given in Figure S6.¹³ A small deviation with respect to crystallographic data is observed for the side chain of Arg331, which moves closer to the phosphate group of the substrate (see Figure S6).

The barriers for the C−C bond formation (TS₁) and the deprotonation (TS₂) steps are very close (17.9 and 18.0 kcal/mol, respectively), and it is therefore not possible to determine on the basis of the calculations which step is the rate-limiting one for this part of the reaction. Since there are no experimental rate constants available for human PAICS, a direct comparison of the calculated barriers with experiments is not possible either. However, kₐₚ values of 32 and 77 s⁻¹ have been measured for the AIRc domain of chicken PAICS and for type II PurE of Treponema denticola, respectively.¹³º These values correspond to a barrier of ca. 15 kcal/mol, which is in good agreement with the barriers calculated here.

The calculations highlight the catalytic role of His303 as a general base in the second step of the reaction. Inspection of the AIRc active site shows that this residue is the only one that can act as a proton acceptor in the proximity of the substrate. Consistently with this observation, this histidine is universally conserved in all class I and class II PurE enzymes.¹³⁻⁴ The importance of the His303 residue for the carboxylation of AIR is also in line with functional complementation experiments carried out using human PAICS mutants introduced into a ΔpurK strain of Escherichia coli, where the introduction of the His303Tyr mutation was linked to the absence of bacterial
Similar experiments were also done with chicken PAICS, where the Ser302Ala/His304Tyr double mutant (corresponding to human PAICS residues Ser301 and His303) abolished the complementation. Furthermore, inactivation of the homologous type I PurE mutase from *E. coli* has been observed in the case of active site His mutations to Asn, Gln, and Trp in mutational experiments combined with enzymatic and functional complementation assays. Similar experiments conducted on PurE-I from *A. aceti* showed complete inactivation of the enzyme when the His was mutated to Asn, Phe, Ala, or Ser, while only traces of activity were obtained when it was mutated to Asp and Gln.

In addition to the mechanism reported in Scheme 1, we have also investigated two additional mechanistic proposals. It has been proposed that the carboxylation reaction proceeds via an ylide mechanism, i.e., starting with the AIR substrate being in the ylide form, with a protonated N and a deprotonated C. This proposal was put forward on the basis of evidence from nonenzymatic carboxylation of AIR. To examine this mechanism, we calculated the energy of the proposed ylide intermediate both in solution and inside the active site model. It turns out that it is as much as 44 kcal/mol higher than the AIR substrate in the solution and 41 kcal/mol higher in the enzyme (see Figure S7). These results are thus sufficient to rule out the ylide mechanism. This is consistent with previous studies on PurE intermediate analogs and also with the study on the prokaryotic class II PurE from *T. denticola* that suggested that AIR does not get protonated at the N position at neutral pH and remains a hydrogen bond acceptor of the conserved Ser301 during the entire carboxylation reaction.

Finally, we also carried out additional calculations to investigate whether the carboxylation of AIR can take place through the reaction with bicarbonate (HCO$_3^-$) instead of CO$_2$. The calculations showed that this mechanistic scenario is also associated with very high energies and can be ruled out (see Figure S8 for details).

To summarize this section, we can conclude that the carboxylation occurring via the isoCAIR mechanism is associated with feasible energy barriers. We anticipate that the results and the mechanistic details provided by the current calculations for human PAICS can be extended to both class I and class II PurE enzymes in general.

### 3.2. Phosphorylation–Condensation in the SAICARs Active Site

After the carboxylation, it has been suggested that CAIR moves from the AIRc to the SAICARs active site without being released into the solution, thus becoming the substrate of the phosphorylation–condensation reaction. As observed in other enzymes with multiple catalytic sites, this often takes place via an intermediate tunnel system that connects the active sites. In the case of human PAICS, the channel was recently recognized by inspection of the octameric structure of the bifunctional enzyme.

In the SAICARs catalytic pocket, CAIR is first phosphorylated in a reaction with ATP before the reaction with Asp, acting analogously to adenylsuccinate synthetase. Although the addition of the individual substrates to the
active site has been shown to be random,\textsuperscript{15} it has also been suggested that Asp and ATP saturate the SAICARs active site before the binding of CAIR\textsuperscript{15} and that the recognition of ATP by SAICARs might facilitate the binding of CAIR\textsuperscript{15}. In the model, we include all three components (ATP, CAIR, and Asp) from the start of the reaction. As discussed above, three Mg ions are included in the model in analogy with the SAICARs:ADP:CAIR structure from \textit{E. coli}.\textsuperscript{13}

In the optimized E:ATP:CAIR:Asp complex, the carboxyl group and the N\textsuperscript{3} of the aminooimidazole ring of the CAIR substrate coordinate Mg\textsubscript{C}. It is likely that CAIR associates with Mg\textsubscript{C} on its way from the AIRc to the SAICARs active site, because SAICARs recognizes the substrate in a complex with magnesium.\textsuperscript{13} The Mg\textsubscript{C} ion is additionally coordinated by Glu97, Asp137, and two water molecules. The Glu97 and Asp137 residues are further bound to Mg\textsubscript{B} Asp137 bridging the two ions in a \(\mu(1,1)\) fashion, while Glu97 bridging in a \(\mu(1,3)\) fashion. The \(\gamma\)-phosphate of ATP and two water molecules complete the coordination sphere of Mg\textsubscript{B}. The ATP, which coordinates to Mg\textsubscript{A} with the oxygens from the \(\alpha\), \(\beta\), and \(\gamma\)-phosphates, is involved in hydrogen-bonding interactions with the backbones and side chains of a number of amino acids, such as Lys19, Asn43, and Lys131 (see Figure 5). The above interactions are preserved in all intermediates and transition states throughout the reaction and match well with those observed in X-ray structures of ternary complexes PAICS:AMP-PNP:SAICAR\textsuperscript{13u} and \textit{E. coli} SAICARs:ADP:CAIR,\textsuperscript{13} with two and three Mg ions, respectively. Important distances of the coordination spheres of the Mg ions are provided in Table S1.

The two carboxylate moieties of the Asp substrate are involved in hydrogen bonds with the Arg232, Lys228, Thr40, and Lys191 residues, which constitute the binding region of the substrate, as also observed in the SAICARs:Asp complex of \textit{Saccharomyces cerevisiae} (PDB 2CNU). The amino group of Asp interacts with the N\textsuperscript{3} amino group of CAIR, suggesting that the latter can play a role in the recognition of Asp. This is consistent with a previous hypothesis that SAICARs enzymes recognize the amino group of Asp to discriminate the substrate from other dicarboxylic acids, like the intermediates of the citric acid cycle.\textsuperscript{15} Interestingly, the calculations show that such recognition is mediated through the CAIR substrate rather than through the active site residues.

It is interesting to mention here that we also considered an alternative binding mode of the ATP in the E:ATP:CAIR:Asp complex where Mg\textsubscript{B} is coordinated by two phosphates (E:ATP:CAIR:Asp’; Figure S9). Such binding mode would be analogous to the crystal structures of enzymes having a similar fold but binding only two Mg ions (\textit{e.g.}, inositol phosphate multikinase and inositol hexakaphosphate kinase, PDB codes SW21 and 4O4D, respectively).\textsuperscript{26} However, the energy was found to be more than 5 kcal/mol higher than the E:ATP:CAIR:Asp complex, mainly due to differences in the hydrogen-bonding network of the \(\alpha\)- and \(\beta\)-phosphate groups (see Figure S9).

The present calculations show that the phosphorylation proceeds through a concerted S\textsubscript{N}2 attack of the carboxylate moiety of CAIR on the \(\text{P}^\nu\) of ATP, in agreement with the proposal on the phosphorylation of CAIR for the \textit{E. coli} SAICARs.\textsuperscript{13} The transition state for this step (TS\textsubscript{3}, Figure 5) has an energy of +10.3 kcal/mol relative to the E:ATP:CAIR:Asp complex (see Figure 6). The forming \(\text{O}^\nu\) and the breaking \(\text{P}^\nu\)–\(\text{O}^\nu\) bonds at TS\textsubscript{3} are 2.08 and 2.24 Å, respectively (Figure 5).

Interestingly, initial attempts to locate TS\textsubscript{3} using models that did not include the Mg\textsubscript{A} and Mg\textsubscript{B} ions and the amino acids composing the ATP binding site were not successful and resulted in the return to E:ATP:CAIR:Asp. We attribute such behavior to the absence of the positive charge that stabilizes the negative charges of the oxygen atoms of the \(\alpha\)- and \(\beta\)-phosphates of the nascent ADP. A large active site model was therefore necessary to study the phosphorylation reaction.

In INT\textsubscript{2}, which is 6.3 kcal/mol below the reactants, the phosphorylated CAIR (\textit{phospho–CAIR}) is formed and can react with Asp in the following condensation reaction. In this intermediate, the two carboxylate groups of Asp are engaged in hydrogen bonds with Thr40, Lys191, Lys228, and Arg232 (see Figure S10). The first step of the condensation reaction (TS\textsubscript{4}), in which the formation of the \(\text{N}_\text{Asp}^\cdot\text{C}^\cdot\text{CAIR}\) bond takes place, has a barrier of 16.3 kcal/mol, with respect to INT\textsubscript{2}, and the resulting tetrahedral intermediate INT\textsubscript{3} is 15.0 kcal/mol higher than INT\textsubscript{2}. The protonated \(\text{N}_\text{Asp}^\cdot\) in INT\textsubscript{3} is involved in the hydrogen-bonding interaction with the oxygen of the \(\gamma\)-phosphate. Importantly, the formed oxyanion is stabilized by binding to both the Mg\textsubscript{C} and Mg\textsubscript{B} ions (see structures in Figure S10).

In TS\textsubscript{5}, the last step of the condensation reaction, the dephosphorylation takes place by a concerted cleavage of \(\text{C}^\cdot\text{CAIR}^\nu\cdot\text{OP}^\nu\) bond and deprotonation of \(\text{N}_\text{Asp}^\cdot\) forming the amide bond. The calculated barrier for this step is 21.7 kcal/mol relative to INT\textsubscript{2}. The products SAICAR and HPO\textsuperscript{4}\textsuperscript{−} (\(\text{P}_\text{i}\)) are finally obtained in the complex E:ADP:SAICAR:P\(_i\), with a favorable thermodynamics of \(-7.1\) kcal/mol relative to E:ATP:CAIR:Asp (see Figure 6).

Comparison of the optimized geometry of E:ADP:SAICAR:P\(_i\), with the available crystal structure of PAICS:AMP-PNP:SAICAR\textsuperscript{13u} reveals some interesting differences that are mainly due to the lack of Mg\textsubscript{B} and P\(_i\) in the crystal structure (superposition of the two structures is reported in Figure S11). The P\(_i\) moiety in E:ADP:SAICAR:P\(_i\), coordinates to all three Mg ions, which results in a different conformation of the SAICAR product in the active site as compared to the crystal structure. For example, the carboxylate groups of SAICAR are involved in hydrogen-bonding interactions with Lys228 and Thr40, which is not the case in the crystal structure. Consistently with this, the previous crystallographic analysis showed that the N-succinyl moiety of SAICAR is rather flexible, which might also be important for the release of the product from the active site.\textsuperscript{13u}
The calculated energy profile in Figure 6 reveals that the final step, i.e., the dephosphorylation (TS5), is the rate-determining step of the phosphorylation–condensation reaction. The overall barrier from INT2 (21.7 kcal/mol) is in quite good agreement with the available kcat of 3 s−1, which corresponds to a barrier of ca. 17 kcal/mol.26

We have further examined the possibility of a phosphorylation reaction taking place through a transfer of the γ-phosphate from ATP first to Glu193, then to Glu97, and finally to CAIR, in accordance with a proposal based on the crystal structure of SAICARs from Streptococcus pneumoniae.13 The calculations show that already the first intermediate in such a mechanism, i.e., the phosphorylation of Glu193, is 16.3 kcal/mol higher than E:ATP:CAIR:Asp (see Figure S12). This mechanistic proposal can thus be ruled out since it is associated with a much higher energy than the direct phosphorylation of CAIR via TS3.

We have also tested an alternative mechanistic proposal in which the condensation reaction takes place before the phosphorylation (see Figure S13).13 The first intermediate in such a mechanism corresponds to a geminal diolate on the C4 center of CAIR. Geometry optimization of such species was not possible and always returned to the E:ATP:CAIR:Asp complex. However, a constrained optimization with a fixed C4−NAsp−Asp has an energy of more than 30 kcal/mol higher than E:ATP:CAIR:Asp, i.e., more than 15 kcal/mol higher in energy than the highest transition state (TS5). This result rules out the possibility of the condensation reaction taking place prior to the phosphorylation.

The current calculations show that the Mg ions catalyze the reaction by binding the CAIR and ATP moieties and stabilizing the negative charges that arise at the transition states and intermediates of the reaction. This role of the Mg ions can be linked to enzyme kinetics studies indicating that a higher number of magnesium cations than those needed for the saturation of ATP are required for catalysis.13,15

Finally, it is interesting to point out that the proposed mechanism for the phosphorylation–condensation reaction (Scheme 1) does not require the direct involvement of any SAICARs active site residues. In related reactions, active site residues might act as nucleophiles or general acids/general bases. Instead, the amino acids of the SAICARs active site bind the substrates and provide the environment necessary to coordinate the Mg ions that facilitate the reaction.

4. CONCLUSIONS

In the present work, we have employed the quantum chemical cluster approach to study the detailed reaction mechanism of the bifunctional human PAICS enzyme. This enzyme is essential for the de novo purine biosynthesis and therefore constitutes a target for new cancer therapies.

The recently solved crystal structures of human PAICS in complex with CAIR, SAICAR, and AMP-PNP13b were used to design two models of the AIRc and SAICARs active sites, consisting of 269 and 369 atoms, respectively. Using these models, different mechanistic proposals were examined and compared in terms of energetic feasibility.

It is shown that in the case of carboxylation, the reaction proceeds via the “isoCAIR mechanism”, where CO2 undergoes a nucleophilic attack of C4 of AIR. His303 acts then as a general base to form the CAIR product.

With regard to the phosphorylation–condensation reaction occurring in the SAICARs domain, the calculations show that the CAIR substrate must first be phosphorylated to react with Asp. The phosphorylation of CAIR takes place directly from ATP, by an S2 mechanism. The SAICAR product is formed through a two-step condensation mechanism, involving first a C=N bond formation between Asp and the phospho–CAIR and then the dissociation of Pγ. According to the calculations, the last step is rate-limiting for the entire reaction. All three Mg ions are demonstrated to be directly involved in the substrate binding and the stabilization of the transition states and intermediates along the phosphorylation–condensation reaction.

We believe that the insights provided by the current calculations will be an important asset in the continued design of new, and the optimization of existing, inhibitors of human PAICS for applications in anticancer therapies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c05072.

Additional results discussed in the text; absolute energies; and Cartesian coordinates of optimized geometries (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AIR aminoimidazole ribonucleotide
AIRc AIR carboxylase
AMP-PNP adenosine 5′-(β,γ-imido)triphosphate
CAIR carboxyaminoimidazole ribonucleotide
PAICS phosphoribosylaminomimidazole carboxylase and phosphoribosylaminomimidazole succinocarboxamide synthetase
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