The Kinetic Mechanism of the Human Bifunctional Enzyme ATIC (5-Amino-4-imidazolocarboxamide Ribonucleotide Transformylase/Inosine 5'-Monophosphate Cyclohydrolase)

A SURPRISING LACK OF SUBSTRATE CHANNELING*

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5-Amino-4-imidazolocarboxamide ribonucleotide transformylase/IMP cyclohydrolase (ATIC) is a bifunctional protein possessing two enzymatic activities that sequentially catalyze the last two steps in the pathway for de novo synthesis of inosine 5'-monophosphate. This bifunctional enzyme is of particular interest because of its potential as a chemotherapeutic target. Furthermore, these two catalytic activities reside on the same protein throughout all of nature, raising the question of whether there is some kinetic advantage to the bifunctionality. Rapid chemical quench, stopped-flow absorbance, and steady-state kinetic techniques were used to elucidate the complete kinetic mechanism of human ATIC. The kinetic simulation program KINSIM was used to model the kinetic data obtained in this study. The detailed kinetic analysis, in combination with kinetic simulations, provided the following key features of the enzyme reaction pathway. 1) The rate-limiting step in the overall reaction (2.9 ± 0.4 s⁻¹) is likely the release of tetrahydrofolate from the formyltransferase active site or a conformational change associated with tetrahydrofolate release. 2) The rate of the reverse transformylase reaction (6.7 s⁻¹) is 2–3-fold faster than the forward rate (2.9 s⁻¹), whereas the cyclohydrolase reaction is essentially unidirectional in the forward sense. The cyclohydrolase reaction thus draws the overall bifunctional reaction toward the production of inosine monophosphate. 3) There was no kinetic evidence of substrate channeling of the intermediate, the formylaminoimidazole carboxamide ribonucleotide, between the formyltransferase and the cyclohydrolase active sites.

5-Amino-4-imidazolocarboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC) is a bifunctional enzyme that catalyzes the penultimate and final steps in the de novo purine nucleoside biosynthetic pathway. AICAR formyl transferase (AICARFT) catalyzes the transfer of the formyl group from a reduced folate cofactor, N⁷-thyrimidylate (10-IF₇₋₇), to the exocyclic amino group of the purine precursor, AICAR, to form 5-formylaminoimidazole-4-carboxamide ribonucleotide (FAICAR). IMP cyclohydrolase (IMPCH) catalyzes the subsequent ring closure reaction to form IMP and a molecule of water (Fig. 1). These activities are located in separate domains of the ATIC protein. The AICARFT activity is located in a larger C-terminal region, and the IMPCH activity resides in a smaller N-terminal region (1).

Two forms of ATIC have been found in human cells. The form described by Rayl et al. (1), which we term hATIC-a, differs from the form (hATIC-b) described by Sugita et al. (2) and Yamauchi et al. (3) only in the six N-terminal amino acids and a single amino acid substitution at position 165. Because of the likelihood that hATIC-b is the predominant form expressed in human tissues (2), this isoform was used to perform all of the experiments described here.

Interest in ATIC stems in part from its potential as a chemotherapeutic target. Glycinamide ribonucleotide transformylase, the third enzyme activity in the de novo purine biosynthetic pathway, and the AICARFT activity of ATIC each require the same reduced folate cofactor, which makes them potential targets for the antifolate class of chemotherapeutic agents. (6R)-5,10-Dideazatetrahydrofolate (DDATHF, Lomefox) is a potent antiproliferative agent that functions through the inhibition of glycinamide ribonucleotide transformylase. The (6S) diastereomer of DDATHF inhibits both glycinamide ribonucleotide transformylase and AICARFT activities (4–7). The actions of certain non-steroidal anti-inflammatory agents, as well as the anti-inflammatory effects of the antifolate methotrexate, have been attributed in part to the inhibition of AICARFT (8–10).

ATIC is the only enzyme involved in the de novo synthesis of purine nucleotides that is bifunctional in every organism studied. This apparent evolutionary conservation raises the question of whether there may be a functional advantage to this structural arrangement. Perhaps the intermediate, FAICAR, is transferred directly from the AICARFT site to the IMPCH site.
without diffusing through bulk solution. The process of the direct transfer of an intermediate has been termed substrate channeling. This phenomenon has been demonstrated to occur with several multifunctional enzymes (11–14) as well as with complexes of enzymes that associate but are not covalently connected (15). The advantages conferred by substrate channeling may include the maximization of efficiency in the use of metabolites in a pathway and the transfer of very unstable or volatile products that would have a very short half-life in bulk solution.

Among the best characterized examples of substrate channeling are those for which there is both kinetic evidence of channeling and a clear structural basis for the process (16). Such examples include tryptophan synthase (11), the bifunctional thymidylate synthase/dihydrofolate reductase from *Leishmania major* (12), and carbamoyl phosphate synthetase (17).

There are at least two cases of apparent substrate channeling in the *de novo* purine nucleotide biosynthetic pathway. The first enzyme in the pathway, glutamine phosphoribosylpyrophosphate amidotransferase, catalyzes the hydrolysis of glutamine to glutamate plus NH$_3$ and the transfer of the NH$_3$ to phosphoribosylpyrophosphate, forming phosphoribosylamine. Structural and kinetic studies of the enzyme from *Escherichia coli* have shown that the two steps are carried out at separate sites and that the NH$_3$ is transferred through a transient 20 Å hydrophobic channel (13, 14).

For the second case for which there is evidence of substrate channeling in the *de novo* purine nucleotide biosynthetic pathway occurs between the first and second enzymes in the pathway, glutamine phosphoribosylpyrophosphate amidotransferase and glycaminde ribonucleotide synthetase, respectively. Kinetic evidence suggests that the unstable intermediate, phosphoribosylamine, must be channeled directly from the first enzyme to the second, although attempts to isolate a complex between the two enzymes have thus far been unsuccessful (15).

Bifunctional ATIC seemed to be a strong candidate for substrate channeling based on its conservation of bifunctionality throughout nature as well as its role in a metabolic pathway. The use of steady-state isotope dilution experiments and substrate analogs of AICAR have provided indirect evidence suggesting that channeling of FAICAR does not occur with ATIC (18–20). In this report, we have addressed the question of substrate channeling directly using a transient kinetic approach that includes rapid chemical quench and stopped-flow methodologies. This strategy has several advantages in examining substrate channeling because it allows one to directly monitor chemical catalysis at each active site as well as the transit of the putative intermediate or metabolite from one active site to another through the use of single turnover kinetic analysis. Using this approach, we provide a complete kinetic description of the reaction pathway for the ATIC enzyme as well as definitive evidence for the lack of substrate channeling.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Human ATIC*—The previously described plasmid, pETATIC-b (21), was transformed into *E. coli* Tuner(DE3)(Novagen) by heat shock. *E. coli* transformants were grown in 2YT bacterial media with 50 µg/ml kanamycin at 37 °C to an A$_{600}$ value of 0.6–0.8. The cultures were then induced with 400 µM isopropyl-1-thio-β-D-galactopyranoside and grown at 30 °C for 3 h. The cells were harvested by centrifugation, washed with 0.85% NaCl, and either stored at −80 °C or used immediately. The purification of human ATIC was carried out as described previously (21).

*Synthesis of [3H]FAICAR—* [3H]FAICAR was purchased from Moravek Biochemicals and converted enzymatically to [3H]FAICAR. hATIC (100 µM) was incubated under single turnover conditions (50 µM [3H]FAICAR and 300 µM 10-fFH$_4$). Samples of the reaction mixture were collected at time points early in the course of the reaction (100 ms). These samples were pooled and lyophilized to dryness. The reaction products were separated by HPLC using a Whatman 10-µm SAX anion exchange column eluted isocratically with 10 mM K$_2$HPO$_4$, pH 2.5, at 1 ml/min. The retention times for standards were as follows: AICAR, 8.1 min; FAICAR, 12.9 min; and IMP, 15.9 min. The [3H]FAICAR peak was collected, lyophilized, and redisolved in a suitable amount of 50 mM ammonium acetate to be further purified by reverse phase HPLC.

The reverse phase HPLC was performed using a 7-µm Adsorbosphere nucleotide-nucleoside reverse phase column (Alltech Associates) eluted isocratically with a mobile phase consisting of 50 mM ammonium acetate, pH 7.0, 5 mM tetraethylammonium hydroxide, and 10% methanol at 1 ml/min. The retention time for the FAICAR standard was 6.9 min, whereas AICAR and IMP standards co-eluted at 10 min. The [3H]FAICAR peak was collected, lyophilized, and redisolved in a suitable amount of 50 mM ammonium acetate to be further purified by reverse phase HPLC.

**Rapid Chemical Quench Experiments—**Rapid chemical quench experiments were performed using a PerkinElmer Lambda 2 spectrophotometer and PerkinElmer PECCSS computerized spectroscopy data acquisition software. All reaction mixtures were 500 µl in volume and were carried out in 1-cm pathlength quartz cuvettes at room temperature unless otherwise noted.

**Steady-state Spectrophotometric Assays—**All steady-state spectrophotometric assays were performed using a PerkinElmer Lambda 2 spectrophotometer and PerkinElmer PECCSS computerized spectroscopy data acquisition software. All reaction mixtures were 500 µl in volume and were carried out in 1-cm pathlength quartz cuvettes at room temperature unless otherwise noted.

For the standard AICAR assay, reaction mixtures contained final concentrations of 66 mM Tris-HCl, pH 7.4, 50 mM KCl, 100 µM AICAR (Sigma), and 300 µM 10-fFH$_4$. The reaction was initiated by the addition of hATIC (typically 10–500 nM final concentration). The reactions were monitored by observing the formation of THF at 298 nm by a method described previously (22, 23). 10-fFH$_4$ was synthesized from (6S)-5-formyl-5,6,7,8-tetrahydrofolic acid (Schircks Laboratories) by a previously described method (1, 24, 25).

For the standard reverse AICAR assay, the buffer concentrations were the same as for the forward direction. Substrate concentration ranges were 5–500 µM FAICAR and 10–500 µM THF. The reactions were monitored by observing the disappearance of THF as a decrease in absorbance at 298 nm by the same method as for the forward direction, described above. FAICAR was synthesized from AICAR according to a published procedure (22).

For the standard IMPH assay, reaction mixtures contained final concentrations of 100 mM Tris-Cl, pH 7.5, and 100 µM FAICAR. hATIC was then added to a final concentration of 10–500 nM to initiate the reaction. The reactions were monitored by observing the appearance of IMP as an increase in absorbance at 248 nm (22).

**Rapid Chemical Quench Experiments—**Experiments were carried out using a KinTek® RQF-3 rapid chemical quench apparatus (KinTek Instruments). Reactions were initiated by mixing 15 µl of enzyme with 15 µl of substrate solution containing [3H]FAICAR (labeled nonspecifically by tritium exchange, 10–20 Ci/mmol, Moravek Biochemicals) and unlabeled 10-fFH$_4$, or by mixing 15 µl of enzyme with 15 µl of substrate solution containing [3H]FAICAR in a reaction buffer consisting of 50 mM triethylammonium bicarbonate, pH 8.0, 50 mM KCl, and 5 mM β-mercaptoethanol. In all cases, concentrations cited in the text correspond to concentrations after mixing and during the enzymatic reaction. Reaction times ranged from 3 ms to 2 s. All rapid chemical quench
experiments were performed at 37 °C unless otherwise noted. Reactions were quenched with 67 μl of triethylamine or 67 μl of 0.5 M formic acid and collected in 1.5-mL tubes containing 70 μl of chloroform. The tubes were immediately vortexed and kept on ice until all of the samples in the set were collected. Samples were then centrifuged at 3000 × g for 10 min to separate the aqueous and organic phases. The aqueous phase was transferred to a clean tube, lyophilized, and redisolved in 8 μl of H2O for subsequent TLC separation on POLYGRAM® CEL 300 polyethylenimine/UV254 TLC plates (Macherey-Nagel, GmbH). TLC separation of substrates and products was performed by a modification of the method described previously (18).

Stopped-flow Absorbance—Stopped-flow absorbance experiments were performed with a KinTek® SF-2001 (KinTek Instruments) stopped-flow fluorescence/absorbance apparatus. This apparatus has a 1.5-ms dead time, a 0.5-cm path length, and a thermostatted observation cell that was maintained at 25 °C. For the AICART reaction in the forward direction, 20 μl of hATIC (5–20 μM) was mixed with 20 μl of a substrate solution containing AICAR (5–200 μM) and 10-ffH4 (from 40 μM to 1 mM). For the reverse AICART reaction, 20 μl of hATIC (from 5 to 200 μM) was mixed with 20 μl of a substrate solution containing FAICAR (300 μM) and THF (from 40 μM to 1 mM). Reactions were monitored at 298 nm (absorbance maximum for THF) for the appearance or disappearance of THF on a time scale of up to 2 s. Most experiments were performed under pre-steady-state burst conditions, and an average of 4–5 runs were used for data analysis. The data were collected over a given time interval by a computer using data acquisition and analysis software provided by KinTek instruments. Data were fit to a single exponential or burst equation by nonlinear regression. The combination of rapid chemical quench and stopped-flow methods allowed an accurate interpretation of absorbance signals.

Data Analysis and Kinetic Modeling—The rate constants for individual single turnover rapid chemical quench experiments were determined by fitting the data to a single or double exponential using the curve-fitting program KaleidaGraph (Synergy Software). The dependence of the pre-steady-state burst rate on substrate concentrations was plotted and fit to a hyperbolic equation to obtain the apparent binding constants for AICAR and 10-ffH4. The rate constants and binding constants obtained from these measurements as well as the steady-state parameters determined here as constraints, we then sought to predict a kinetic model that would be consistent with all of the kinetic measurements and possibly provide insights into substrate channeling and/or domain-domain interactions.

The kinetic simulation program KINSIM (26) was used to model the kinetic data. The data from single turnover experiments were entered as x y pairs and were fit by a trial and error process using the rate constants obtained from the exponential fits of single turnover experiments and the binding constants, Km values, and kcat values obtained in this study. The focus of the modeling was to ascertain, with the rate constants obtained experimentally, whether it was necessary to invoke a channeling step to explain any of the kinetic data.

RESULTS

Steady-state Kinetics of hATIC—The steady-state spectrophotometric assays for AICART and IMPCH activities were used to determine kcat and Km values for the substrates of these activities. The results are shown in Table I. The Km values for AICAR and 10-ffH4 were similar to the values previously reported for this enzyme (1, 2). The kcat value for FAICAR at the IMPCH site is necessarily an approximation because the spectrophotometric assay lacks the sensitivity to accurately determine values less than 1 μM (22). The value obtained was similar to previously reported results (1, 22). Steady-state chemical quench techniques were also used to determine kcat values (not shown), and the values obtained were in good agreement with the values obtained spectrophotometrically.

Substrate Binding to the AICART Active Site—It has been previously reported by ourselves (21) and others (20) that the human AICARTF activity exhibits a pre-steady-state burst of FAICAR formation, suggesting that the rate-limiting step is determined at least in part, by a step occurring after chemical catalysis. These results were confirmed by the use of both rapid chemical quench and stopped-flow absorbance. In all cases, the amplitude of the burst phase was nearly equal to the concentration of enzyme used in the experiments, indicating that close to 100% of the AICARTF catalytic sites were active at the ATIC concentrations used in these experiments (20, 21).

To determine the apparent binding constants for AICAR and 10-ffH4 at the AICART active site, a series of pre-steady-state burst experiments was performed to determine the substrate concentration dependence of the pre-steady-state burst rate. Stopped-flow absorbance was used in these burst experiments to monitor the appearance of THF at 298 nm on a short time scale (<1 s) (Fig. 2). This type of experiment was carried out with varying concentrations of one substrate while keeping the other substrate constant under saturating conditions. To examine 10-ffH4 binding, pre-steady-state burst experiments were performed in which AICAR was maintained at a concentration of 200 μM, and the concentration of 10-ffH4 was varied from 40 μM to 1 mM. To examine AICART binding, the 10-ffH4 concentration was kept at 500 μM, and AICAR was varied from 5–200 μM. The exponential rates from the burst curves (fast phase) were plotted versus substrate concentration, and in each case the resulting curve was fit to a hyperbola from which apparent binding constants were directly obtained. The apparent binding constant for 10-ffH4 was found to be 105 ± 7 μM, and for AICAR it was 22 ± 4 μM.

Substrate Binding in the Reverse AICART Reaction—A pre-steady-state burst experiment was performed for the reverse AICART reaction using stopped-flow absorbance. The reaction was initiated by mixing 10 μM hATIC with a substrate solution containing 300 μM FAICAR and 300 μM THF. We monitored the disappearance of THF as a decrease in absorbance at 298 nm as it was converted to 10-ffH4 and FAICAR was converted to AICAR. In the reverse direction, no pre-steady-state burst phase was observed (Fig. 3), suggesting that the rate of chemical catalysis is at least partially rate-limiting.

The steady-state spectrophotometric assay was used to determine the Km for FAICAR and tetrahydrofolate at the AICART active site. The Km for tetrahydrofolate was determined to be 185 ± 27 μM. The Km for FAICAR at the AICARTF active site was estimated to be <3.9 ± 1.3 μM. The spectrophotometric assay was not sensitive enough to detect accurate observed rates for FAICAR concentrations under 5 μM because assays at these lower concentrations are complicated by the simultaneous depletion of FAICAR by the IMPCH activity. A selective inhibitor of the IMPCH activity was not available at the time of these experiments. The maximum steady-state rate of catalysis in the reverse direction was calculated as 6.7 ± 0.6 s−1, which is faster than the steady-state rate in the forward direction (2.9 ± 0.4 s−1).

Investigation of Substrate Channeling—To investigate the possibility of substrate channeling between the two active sites of ATIC, we needed to determine whether the product from the first reaction, FAICAR, is transferred directly from the

### Table I

| Activity | Constant | Value |
|----------|----------|-------|
| AICARTF | Km AICAR | 10 ± 1 μM |
| | Km 10-formylFH4 | 100 ± 9 μM |
| | kcat | 2.9 ± 0.4 s−1 |
| IMPCH | Km FAICAR | 1.4 ± 0.1 μM |
| | kcat | 6.0 ± 0.8 s−1 |
AICARFT site to the IMPCH site and not allowed to dissociate from the enzyme. This was done by a series of three single turnover rapid chemical quench experiments performed as illustrated in Fig. 4.

The first experiment established the rate of catalysis of the AICARFT activity under single turnover conditions as designated in reaction 1 in Fig. 4. hATIC (80 μM) was mixed with excess 10-fFH4 (500 μM) and a limiting amount of [3H]AICAR (20 μM). The decoupling of the bifunctional reaction is complicated by the fact that FAICAR is the single substrate for the IMPCH activity. In this experiment both [3H]FAICAR and [3H]IMP were formed and detected by TLC. To obtain the rate of FAICAR formation, the total concentration of product formed (FAICAR plus IMP, because any IMP present was formed via FAICAR) was plotted versus time, and the resulting curve was fit to a single exponential. The time course for the formation of [3H]FAICAR is shown in Fig. 5. Under these conditions, the rate of catalysis was 62 ± 4 s⁻¹. There was no change in this rate under conditions in which the enzyme concentration was significantly increased, indicating that substrate binding was not rate limiting in these experiments.

The second set of single turnover experiments was designed to examine the IMPCH activity of bifunctional ATIC as designated in reaction 2 in Fig. 4. hATIC (40 μM) was mixed with a limiting amount of [3H]FAICAR (20 μM). The time course for the formation of [3H]IMP is shown in Fig. 6. Interestingly, this curve was biphasic and fit best to a double exponential. The first phase had an amplitude of 8.4 ± 0.8 μM and a rate of 40 ± 6 s⁻¹. This is likely the rate of catalysis in a single turnover of IMPCH activity. The second phase had an amplitude of 10 ± 1 μM and a rate of 2.3 ± 0.7 s⁻¹, which was similar to but slightly slower than the steady-state rate of IMP formation from FAICAR of 6.0 s⁻¹ (Table I).

The third set of single turnover experiments was designed to directly measure the rate of conversion of AICAR to FAICAR at the AICARFT active site and the subsequent conversion of FAICAR to IMP at the IMPCH active site (designated as reaction 3 in Fig. 4). If FAICAR dissociates from the AICARFT active site and rebinds at the IMPCH active site, it is probable that it would be detected as an intermediate in the bifunctional reaction. However, if FAICAR is channeled directly from the AICARFT active site to the IMPCH active site, it is unlikely to accumulate during the time course of the overall bifunctional reaction. In these experiments, which were set up in essentially the same manner as the first set of experiments, hATIC (80 μM)
The hATIC enzyme (40 μM) was mixed with [3H]AICAR (20 μM). The formation of [3H]IMP (●) was monitored by TLC as described under “Experimental Procedures”. The data were fit to a double exponential to provide a rate of 40 ± 6 s⁻¹ for the first phase and a rate of 2.3 ± 0.7 s⁻¹ for the second phase.

was mixed with excess 10-fFH₄ (500 μM) and limiting concentrations of [3H]AICAR (20 μM). In this case, the intermediate FAICAR and the ultimate product, IMP, were plotted separately. The time courses of the disappearance of AICAR and the appearance of the intermediate FAICAR and the product, IMP, are shown in Fig. 7. Under these reaction conditions we observed a large accumulation of the intermediate FAICAR, which was subsequently converted to IMP by the end of the time course. This result strongly suggests that channeling of FAICAR from the AICARFT site to the IMPCH site does not occur.

The time course for the formation of IMP in this experiment was very similar to the IMP formation in a single turnover of IMPCH activity. This curve was also biphasic and was best fit to a double exponential. The first phase had an amplitude of 8.3 ± 2.4 μM and a rate of 30 ± 9 s⁻¹, and the second phase had an amplitude of 10.5 ± 2.1 μM and a rate of 4.1 ± 1.6 s⁻¹.

Finding that the rate of IMPCH catalysis is the same in a single turnover of IMPCH activity and a single turnover of the bifunctional ATIC supports a mechanism that does not involve FAICAR channeling. It also suggests a lack of any domain-domain communication that would result in rate enhancement of the IMPCH activity in the bifunctional ATIC reaction. Finally, this result suggests that the rate-limiting step in the overall bifunctional reaction is the release of THF from the AICARFT active site. Pre-steady-state burst experiments indicated that it is likely that the rate-limiting step of the AICARFT reaction (2.9 ± 0.4 s⁻¹, which is also limiting in the overall bifunctional reaction) occurs after the chemical conversion of AICAR to FAICAR. Because the rate of IMP formation in a single turnover of the bifunctional reaction is not limited by the rate-limiting step of the AICARFT reaction, it is likely that the rate-limiting step is at least partially determined by the release of THF or a conformational change associated with THF release under the conditions studied here.

Kinetic Modeling—The individual rate constants, equilibrium constants, and steady-state parameters measured in this kinetic study are summarized by the mechanism shown in Fig. 8. This mechanism was used to construct the kinetic model for the hATIC bifunctional single turnover experiments using KINSIM (Fig. 7). We have previously reported that the substrate binding to the AICARFT active site follows an ordered sequential mechanism with 10-fFH₄ binding first (27). Attempts were made to confirm this by performing substrate trapping experiments. Unfortunately, these experiments were unsuccessful because of our inability to trap either substrate at the active site. This suggests that the rate constants for the binding and dissociation of one or both substrates may be too fast to be determined by this method. Attempts were also made to directly measure rate constants for substrate binding by stopped-flow fluorescence; however, no intrinsic protein fluorescence change was observed upon the addition of either substrate. Nevertheless, based on our previous results, a justification was provided for a model in which 10-fFH₄ binds first.

To account for the biphasic behavior in the time course of IMP formation in the single turnover experiments, it was necessary to include two populations of IMPCH active sites. This was accomplished by including a ligand bound in 50% of the sites. It was assumed that a ligand bound in the IMPCH active sites would not affect the AICARFT activity in a single turnover. The rate of dissociation of the prebound ligand was taken as 2.3 s⁻¹, which was the rate of the slower phase of the biphasic IMPCH curve from the single turnover experiments. The release of THF was taken as a rate-limiting step in the bifunctional reaction, i.e., 2.9 s⁻¹. There was no need to invoke a channeling step or any rate enhancement due to domain-domain communication to explain any of the kinetic data obtained in this study.

DISCUSSION

The application of rapid chemical quench and stopped-flow absorbance to the study of bifunctional human ATIC has allowed a direct analysis of the kinetics for the conversion of AICAR to IMP. This study has provided definitive evidence that the channeling of FAICAR from the AICARFT active site to the IMPCH active site does not occur. This evidence consists of a few key points. First, the large accumulation of FAICAR in a single turnover of the bifunctional reaction suggests that FAICAR dissociates from the AICARFT site and rebinds at the IMPCH site. In cases wherein channeling has been proven (11, 12), the channeling step is very fast (>1000 s⁻¹), and there is very little or no detectable amount of the intermediate product observed in a single turnover.

A second important result was that the rate of formation of
IMP was the same in the bifunctional reaction (40 ± 6 s⁻¹) as it was in the single turnover of IMPCH activity when FAICAR came from the bulk solution (30 ± 9 s⁻¹). In addition to lending further evidence against a substrate channeling hypothesis, this also suggests a lack of any domain-domain communication that would cause rate enhancement of the second activity as has been shown to occur in some proven examples of substrate channeling.

Interestingly, the time course for the formation of IMP in a single turnover of IMPCH activity was found to be biphasic and was best fit to a double exponential. This was also the case for the IMP formation in a single turnover of the bifunctional reaction. It seems likely that the faster phase is the actual rate of IMPCH catalysis. There are at least two possible explanations for the observation of a second, slower phase. One possibility is that there are two populations of IMPCH active sites with different conformations. In this case, the faster phase would be due to catalysis by the population of sites in an active conformation. The slower phase could represent catalysis by a population in a less active conformation or the rate of the conformational change from an inactive to an active conformation. A second possibility is that human ATIC contains a ligand bound in a fraction of the IMPCH active sites. If this is the case, the faster phase of the biphasic curve would represent IMPCH catalysis by the unoccupied fraction of active sites, and the slower phase would represent the rate of dissociation for the bound ligand.

Recent crystallographic data by Wilson and co-workers at the Scripps Research Institute (28) suggest that the later possibility is likely to be the case. Avian ATIC was found to crystallize as a dimer with a purine nucleotide-like density in one of the two IMPCH active sites. It was concluded that the ligand must have been acquired during the expression of the enzyme in E. coli because no nucleotides were added during the purification or crystallization procedures. The human ATIC used in our experiments was purified by the same method as the avian ATIC. Experiments are underway to determine the identity of the ligand in the avian structure as well as to investigate whether this is the cause of the biphasic IMPCH activity observed in single turnover experiments with human ATIC.

The kinetic mechanism shown in Fig. 8 summarizes the individual rate and equilibrium constants that were obtained by this study and used as constraints in the modeling performed with KINSIM. When KINSIM was used to construct the models, there was no need to include any steps for channeling or for rate enhancement to explain any of the data obtained in this study. Thus, we conclude that there is no kinetic evidence to support a substrate channeling hypothesis for hATIC. This is consistent with structural studies that show no evidence of a tunnel or cavity connecting the two active sites to form a channel (28). In fact, any potential pathway between the AICARFT and IMPCH sites is apparently precluded by the presence of two overlapping β-loops that lie between the two domains.

We have also ruled out the possibility of domain-domain communication by the finding that there does not appear to be any rate enhancement of the IMPCH activity in a single turnover of the bifunctional ATIC reaction. Occupation of the IMPCH site by a specific inhibitor was previously shown not to have an effect on AICARFT activity (20), which gives further support to this conclusion.

These conclusions lead to the consideration of other potential functional advantages to the conserved bifunctionality of ATIC. As we have again shown here, it has been previously reported (20) that the AICARFT activity favors the reverse reaction in the steady-state (2.9 s⁻¹ forward versus 6.7 s⁻¹ reverse). The IMPCH reaction, however, highly favors the forward direction. Perhaps the advantage of linking the two activities is to draw the equilibrium of the AICARFT reaction toward the formation of FAICAR by subsequently converting the FAICAR to IMP.

Having the two enzyme activities linked guarantees a stoichiometric amount of the IMPCH active sites available to convert any FAICAR formed to IMP. One could argue that the close proximity (~50 Å apart) (28) of the two active sites provides an increased local concentration of IMPCH active sites. Szabados and Christopherson (19) have performed theoretical calculations that suggested that the local concentration of FAICAR at the IMPCH active sites was higher than the FAICAR in bulk solution (added exogenously), based on the results of their steady-state experiments.

Another possible advantage to the bifunctionality of ATIC is more structural in nature. We have recently shown that in solution hATIC exists in a monomer/dimer equilibrium with a dissociation constant of 240 nM at 4 °C and that the dimeric form is required for AICARFT activity (21). The crystal structure of avian ATIC has been described as an “extensively intertwined” homodimer with a considerable dimer interface for a protein of its size burying 4929 Å² per monomer, of which the IMPCH domain makes up 38% (28). With such a large percentage of the dimer interface from the IMPCH domain, it is possible that the physical linking of these two activities on the same polypeptide helps to keep the protein in the dimeric form required for AICARFT activity. To test the possibility that there is a kinetic or structural advantage to having AICARFT and IMPCH activities physically linked, we plan to perform reconstitution experiments with the AICARFT and IMPCH truncation mutants that we have described previously (1).

In conclusion, we have provided definitive kinetic evidence that substrate channeling does not occur with bifunctional human ATIC. We have also provided a detailed kinetic analysis of this enzyme, which can provide a basis for studies of catalytic mechanism as well as aid in the design characterization of potential inhibitors of these important enzyme activities.

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Kinetic Mechanism of Human ATIC

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