Guanidine Derivatives Rescue the Arg418Ala Mutation of *Tritrichomonas foetus* IMP Dehydrogenase†

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ABSTRACT: IMP dehydrogenase (IMPDH) catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) and the reduction of NAD^+. The reaction involves formation of an E−XMP* covalent intermediate; hydrolysis of the E−XMP* intermediate is rate-limiting and requires the enzyme to adopt a closed conformation. Arg418 appears to act as the base that activates water for the hydrolysis reaction [Guillen-Schlippe, Y. V., and Hedstrom, L. (2005) *Biochemistry 44*, 11700−11707]. Deprotonation of Arg418 also stabilizes the closed conformation. Here we show that guanidine derivatives rescue the activity of the Arg418Ala variant. Amines and imidazole do not rescue. The rescue reaction appears to be saturable, with the values of K_R ranging from 40 to 400 mM. The value of k_{rescue} for the best rescue agents approaches the value of k_{cat} for the reaction of the wild-type enzyme. Guanidine derivatives also rescue the activity of the Arg418Ala/Tyr419Phe variant. Multiple-inhibitor experiments suggest that the guanidine derivatives do not restore the equilibrium between open and closed conformations. Therefore, rescue agents must accelerate the hydrolysis of the E−XMP* intermediate. The rate of the rescue reaction increases with an increase in pH, consistent with the hypothesis that the reaction involves neutral guanidine. A solvent D_2O isotope effect is observed at low concentrations of the rescue agent, consistent with rate-limiting transfer of a proton from water. The value of $k_{cat\ rescue}/K_R^{base}$ correlates with the pK_a of the guanidine derivative (Bronsted coefficient $\beta \sim 1$). These results suggest that proton transfer from water to guanidine is almost complete in the transition state.

The first committed and rate-limiting step in guanine nucleotide biosynthesis is the oxidation of IMP[1] to XMP with the concomitant reduction of NADH catalyzed IMP dehydrogenase (IMPDH). This enzyme is a target for immunosuppressive (*1*), antiviral (*2*), and cancer (*3*) drugs and may also be a target for antimicrobial chemotherapy (*4*, *5*). The reaction occurs in two steps (Scheme 1): (1) oxidation of IMP to form an E−XMP* covalent enzyme intermediate and NADH and (2) hydrolysis of E−XMP* to produce XMP. A large conformational change separates the two steps: after NADH departs, a mobile flap moves into the vacant dinucleotide site. The conformational change places Arg418 and Tyr419 in the nicotinamide subsite near E−XMP*, where they interact with Asp261 (*6*). Arg418 and Tyr419 are within hydrogen bonding distance of the putative catalytic water in a transition-state analogue complex (*6*). Substitutions of Arg418 and Tyr419 decrease the rate of hydrolysis of E−XMP* but do not affect the first step of the reaction. Many inhibitors compete with the flap, so the conformational change is an important determinant of drug sensitivity (*5*, *7*).

Arg418 may act as the base that activates water (*8*, *9*). Substitution of Arg418 decreases the rate of hydrolysis of E−XMP* by a factor of 500, while substitution of Tyr419 decreases the rate of hydrolysis by a factor of only 20. These substitutions also perturb the conformational change, although this effect cannot account for the decrease in the rate of hydrolysis. Both the conformational change and the hydrolysis reaction are modulated by a residue with a pK_a of ∼8. Substitution of Tyr419 does not change the pH dependence of either the conformational change or the hydrolysis reaction. In contrast, the conformational change becomes pH-independent when Arg418 is substituted with Gln. Lys can replace the function of Arg418 in the hydrolysis reaction but does not stabilize the closed conformation. The simplest explanation for these observations is that Arg418 serves as the base that activates water in the IMPDH reaction. Although several other enzymes are believed to utilize Arg residues as a base, this conclusion is controversial because Arg residues usually have a pK_a of ∼13 which would seem to preclude this role (*10*).

Chemical rescue experiments, in which a small molecule replaces a protein residue, have often proven to be useful tools for delineating the role of active site residues. Perhaps the best example of such experiments is the work of Toney and Kirsch (*11*), in which amines rescue the activity of a Lys-to-Ala mutation of aspartate aminotransferase. A cor-

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[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; XMP, xanthosine 5′-monophosphate; LDH, lactic dehydrogenase; MZP, mizoribine monophosphate; ADP, adenosine 5′-diphosphate; tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide; DTT, dithiothreitol; TAD, thiazole-4-carboxamide adenine dinucleotide; SIE, solvent isotope effect.

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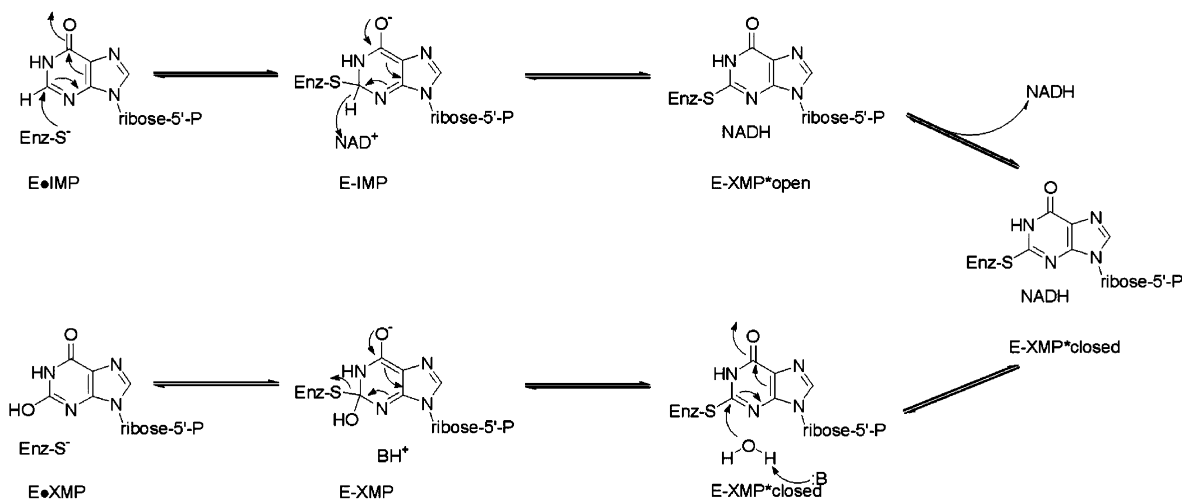
Scheme 1: Mechanism of the IMPDH Reaction



relation was observed between the efficiency of the rescue agent and the basicity of the amine rescue agent, providing the first example of Bronsted analysis applied to an enzymatic reaction. While the best correlation included a steric term, it is worth noting that similar results were obtained when the steric factor was omitted [$\beta = 0.3 \pm 0.2$ vs 0.39 ± 0.05 with the steric factor (*11*)]. This β value suggests that the Lys residue has a positive charge of ~0.4 in the transition state.

While not as common as the rescue of Lys mutations by amines, the rescue of Arg mutations by guanidine derivatives has been reported in carboxypeptidase (*12*), ornithine transcarbamylase (*13*), asparagine synthetase (*14*), mandelate dehydrogenase (*15*), and protein tyrosine kinase (*16*). Interestingly, imidazole can also rescue Arg mutations in protein tyrosine kinase and mandelate dehydrogenase, but not in ornithine transcarbamylase, while amines fail to rescue protein tyrosine kinase, carboxypeptidase, mandelate dehydrogenase, and asparagine synthetase. Bronsted analysis of guanidinium rescue of the Arg mutation of ornithine aminotransferase has also been described, although this correlation is difficult to interpret since the pH dependence of the rescue reaction was not reported (*13*).

Here we have investigated the reaction of the Arg418Ala variant of *Tritrichomonas foetus* IMPDH with guanidine derivatives to gain more insight into the role of Arg418 in this reaction. The rate of the rescue reaction increases with pH as expected if the neutral guanidine is the reactive species. With the caveat that the cavity can accommodate very few guanidine derivatives, Bronsted analysis suggests that proton transfer is almost complete in the transition state. A preliminary description of this work has been published (*10*).

MATERIALS AND METHODS

Materials. IMP, ADP, NADH, guanidine hydrochloride, ethylguanidine hydrochloride, ethanolamine hydrochloride, tetramethylammonium chloride, and Tris were purchased from Sigma (St. Louis, MO). Methylguanidine hydrochloride, aminoguanidine bicarbonate, acetamidine hydrochloride, guanylurea sulfate, and hydroxyurea were purchased from Aldrich. Ethylguanidine hydrochloride was purchased from Avocado Research Chemicals Ltd. *N*-Propylamine and butylamine were purchased from Acros Organics. NAD^+ was purchased from Boehringer Mannheim. DTT was purchased from Research Organics, Inc. D_2O, KOD, and DCl were purchased from Cambridge Isotope Laboratories, Inc. LDH (bovine heart) was purchased from ICN Biomedicals, Inc. Tiazofurin was obtained from NCI. Oligonucleotides were purchased from Operon and Integrated DNA Technologies, Inc.

Site-Directed Mutagenesis. A construct designated pTf1 containing the *T. foetus* IMPDH coding sequences in the pKK223-3 plasmid (*17*) was used as a template to construct Arg418Ala/Tyr419Phe using the Quikchange kit (Stratagene, La Jolla, CA). The entire coding sequences were sequenced to ensure that no undesired mutations were introduced.

Expression and Purification. Plasmids containing the mutant IMPDH genes were transformed and expressed in *Escherichia coli* H712 cells, which lack endogenous IMPDH (*18*). Arg418Ala and Arg418Ala/Tyr419Phe were purified as previously described (*8*).

Enzyme Kinetics. Standard IMPDH assays contained saturating concentrations of IMP (from 100 μM to 2 mM) and varying concentrations of NAD^+ in 100 mM KCl, 3 mM EDTA, 1 mM DTT, and 50 mM Tris-HCl (pH 8.0) (assay buffer). All assays were performed at 25 °C. Assays of the Arg418 mutants contained LDH (20 μM) to scavenge NADH to alleviate product inhibition (*8*). Activity was measured by monitoring the fluorescence of NADH ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 460$ nm) on a PerSeptive Biosystems Cytofluor II multiwell plate reader. Rates of NADH production were determined by calibration of the instrument with a standard curve of NADH solution in assay buffer containing LDH. The presence of NAD^+ does not affect the fluorescence of NADH under these conditions. Steady-state parameters with respect to NAD^+ were derived at saturating IMP concentrations by plotting the initial velocity against NAD^+ concentration and fitting it to an equation describing uncompetitive substrate inhibition (eq 1) using SigmaPlot (SPSS, Inc.):

$$v = V_m/(1 + K_a/[NAD^+] + [NAD^+]/K_{ii}) \qquad (1)$$

where v is the initial velocity, V_m is the maximal velocity, K_a is the Michaelis constant, and K_{ii} is the substrate inhibition constant for NAD^+.

Chemical Rescue of Arg418Ala. Guanidine derivatives, urea, thiourea, hydroxyurea, acetamidine hydrochloride,

imidazole, *N*-propylamine, ethanolamine, butylamine, and tetramethylammonium chloride were dissolved in water and adjusted to a pH 8.0 with KOH. The concentration of K^+ (100−800 mM) has no effect on the activity of Arg418Ala. The rescue agents have no effect on the binding of NADH to LDH. *N*-Guanylurea ($pK_a = 3.9$) could not be tested because even low concentrations (25 mM) caused LDH to precipitate. The concentration of NAD^+ was varied at fixed rescue agent concentrations. Initial velocity data were corrected for the rate in the absence of the rescue agent and were fitted to eq 1 using SigmaPlot (SPSS, Inc.) to obtain $V_{max\ rescue}$ values, which were fitted to eq 2 to determine the values of k_{rescue}:

$$V_{max\ rescue}/[E]_{tot} = (k_{rescue}[\text{rescue agent}])/(K_R + [\text{rescue agent}]) \quad (2)$$

A Bronsted plot was constructed by plotting the pK_a values of the rescue agent against $\log(k_{rescue}/K_R^{base})$, where K_R^{base} is the K_R corrected for the free base concentration at pH 8.0. The k_{rescue}/K_R^{base} values were fitted to eq 3 where β is the Bronsted coefficient:

$$\log(k_{rescue}/K_R^{base}) = \beta(pK_a) + C \quad (3)$$

Similar β values are obtained when the values of pK_a are normalized for the number of equivalent protons.

Solvent Deuterium Isotope Effects. Assay buffer, substrates, and aminoguanidine, where appropriate, were prepared in D_2O or H_2O. The pH meter readings were corrected to pD by adding 0.4 (*19*). Activity was assayed at saturating IMP concentrations (from 100 μM to 2 mM), while NAD^+ concentrations were varied appropriately.

Multiple-Inhibitor Kinetics. Multiple-inhibitor experiments with tiazofurin and ADP were performed as described previously (*9*). Reaction mixtures contained IMP (100 μM) and NAD^+ (20 μM). Initial velocities were fitted to eq 4:

$$v = v_0/(1 + [I]/K_i + [J]/K_j + [I][J]/\alpha K_i K_j) \quad (4)$$

where v is the initial velocity, v_0 is the initial velocity in the absence of inhibitor, K_i and K_j are the inhibition constants for inhibitors I and J, respectively, and α is the interaction constant.

RESULTS AND DISCUSSION

Chemical Rescue of Arg418Ala by Guanidine Derivatives. Guanidine rescues the activity of the Arg418Ala enzyme, with a maximum k_{rescue} of 0.13 s^{-1} (Figure 1A). This value is 33-fold greater than the k_{cat} of Arg418Ala, and within 7% of the wild-type value (for comparison, $k_{cat} = 1.7\ s^{-1}$ and 0.004 s^{-1} for the wild-type and Arg418Ala enzymes, respectively (*8*)). This experiment utilizes high concentrations of guanidine (300 mM), so the plateau in Figure 1 may result from a nonspecific inhibitory effect rather than saturation of a guanidine binding site. Guanidine derivatives do not change the affinity of inhibitors (see below), which suggests that the global enzyme structure is not affected. However, it is possible that high concentrations of guanidine perturb the conformation of smaller structural elements such as the flap. The activity of the wild-type enzyme decreases at high guanidine concentrations (Figure 1B), which further suggests that the plateau results from a nonspecific effect rather than saturation of the guanidine binding site.

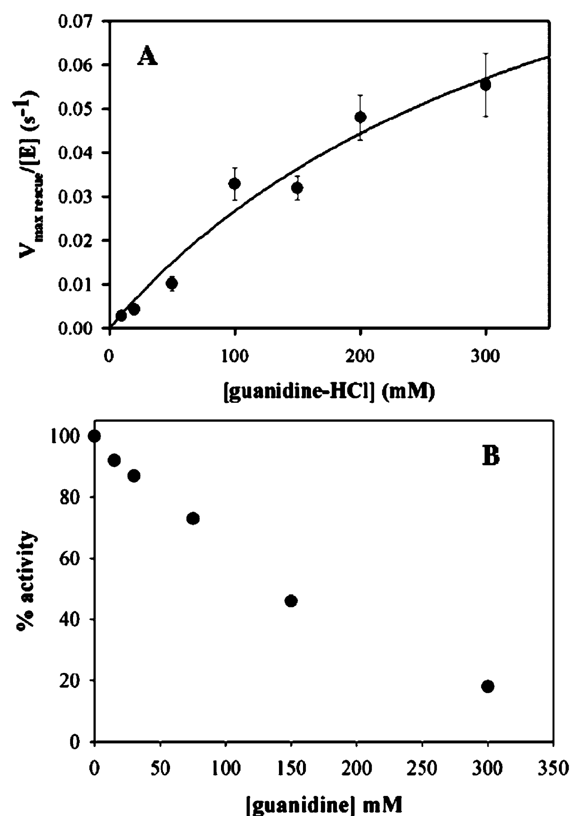
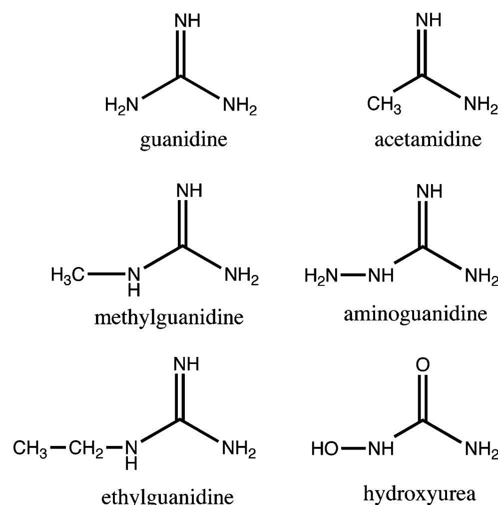


FIGURE 1: Effect of guanidine hydrochloride on the activity of the Arg418Ala and wild-type IMPDHs. (A) The value of $V_{max\ rescue}$ was determined as described in Materials and Methods. (B) Activity was determined in standard assay buffer containing 100 μM IMP and 500 μM NAD^+.

Scheme 2: Structures of Rescue Agents



Several other guanidine derivatives also rescue the activity of Arg418Ala as summarized in Table 1 (see Scheme 2 for structures). Methylguanidine and aminoguanidine exhibited the greatest rate enhancement with the value of k_{rescue} approaching the k_{cat} of the wild type. No rescue was observed with urea (varying concentrations up to 400 mM, $pK_a = 0.2$), thiourea (varying concentrations up to 400 mM, $pK_a = -1.2$), *N*-propylamine (200 mM, $pK_a = 10.6$), ethanolamine (200 mM, $pK_a = 9.4$), butylamine (200 mM, $pK_a = 10.8$), imidazole (400 mM), and tetramethylammonium chloride

Table 1: Chemical Rescue of Arg418Ala and Arg418Ala/Tyr419Phe[a]

enzyme	compound	E_s	pK_a	K_R (M)	k_{rescue} (s^{-1})	k_{rescue}/K_R (s^{-1} M^{-1})	k_{rescue}/k_{cat}	k_{rescue}/K_R^{base} (s^{-1} M^{-1})
Arg418Ala	guanidine	0	13.6[c]	0.39 ± 0.15	0.13 ± 0.03	0.34 ± 0.05	33	(1.3 ± 0.9) × 10^5
	methylguanidine	−1.24	13.4[c]	0.19 ± 0.13	0.35 ± 0.18	2.3 ± 0.4	88	(5.7 ± 1.0) × 10^5
	ethylguanidine	−1.31	13.3[d]	0.040 ± 0.018	0.0094 ± 0.0010	0.23 ± 0.05	10	na[f]
	acetamidine		12.5[c]	0.29 ± 0.13	0.067 ± 0.016	0.23 ± 0.05	17	(3.0 ± 0.7) × 10^3
	aminoguanidine	−0.61	11.0[d]	0.080 ± 0.054	0.33 ± 0.12	4 ± 1	82	(4 ± 1) × 10^3
	hydroxyurea		10.6[e]	0.05*	≥0.0009[b]	0.015 ± 0.012	≥0.25	6 ± 5
Arg418Ala/Tyr419Phe	aminoguanidine	−0.61	11.0[d]	0.2*	≥0.0020 ± 0.0007[b]	na[f]	≥1000	na[f]

[a] E_s is the Taft steric parameter (*26*). The values of K_R and k_{rescue} were determined as described in Materials and Methods. K_R is the concentration of the rescue agent at which half of the maximal k_{rescue} was observed. [b] Measured at the highest concentration that was tested. Asterisks denote the highest concentration that was tested. [c] pK_a values from ref *27*. [d] pK_a value from ref *13*. [e] pK_a values from ref *28*. [f] Not applicable.

Table 2: Rescue of Arg418Ala IMPDH by Guanidine Derivatives[a]

enzyme	rescue agent	SIE	α	K_i (mM) tiazofurin	K_i (mM) ADP
wild-type	none	1.6 ± 0.08	0.007 ± 0.002		
	150 mM guanidine	nd[b]	∼0.1	nd[b]	nd[b]
Arg418Ala	none	4.8 ± 2.0	0.45 ± 0.31	2.4 ± 0.5	1.7 ± 0.5
	10 mM aminoguanidine	4.2 ± 1.3	nd[b]	nd[b]	nd[b]
	25 mM aminoguanidine	2.9 ± 0.9	0.27 ± 0.17	3 ± 1	2.0 ± 0.3
	200 mM aminoguanidine	0.88 ± 0.33	0.61 ± 0.27	5.0 ± 0.8	1.8 ± 0.3
	25 mM methylguanidine	5.0 ± 1.2	nd[b]	nd[b]	nd[b]
	35 mM methylguanidine	3.7 ± 1.0	nd[b]	nd[b]	nd[b]
	100 mM methylguanidine	nd[b]	0.41 ± 0.13	2.4 ± 0.3	2.2 ± 0.3

[a] SIE and multiple-inhibitor analysis of the rescue reaction. Conditions as described in Materials and Methods. The parameters for the wild-type and Arg418Ala enzymes in the absence of the rescue agent are from ref *8* and are included for comparison. The apparent values of K_i for tiazofurin and ADP are derived from eq 4. [b] No data.

(100 mM). The failure of amines to rescue is somewhat surprising given that the Arg418Lys substitution is active (*9*), although, as noted above, amines also fail to rescue other Arg mutations. KCl (100−800 mM) also fails to increase the activity of Arg418Ala, which indicates that rescue cannot be attributed to changes in ionic strength.

Effective Concentration of Arg418. The rescue reaction can be used to estimate an effective concentration for Arg418 at the active site: $k_{HOH}/(k_{rescue}/K_R) \sim 2$ M using methylguanidine as the rescue agent. This value is on the low side for effective concentrations of active site residues; effective concentrations of 30−55 M can be calculated for the rescue of Arg mutations by guanidine derivatives in ornithine transcarbamylase and carboxypeptidase A (*12*, *13*).

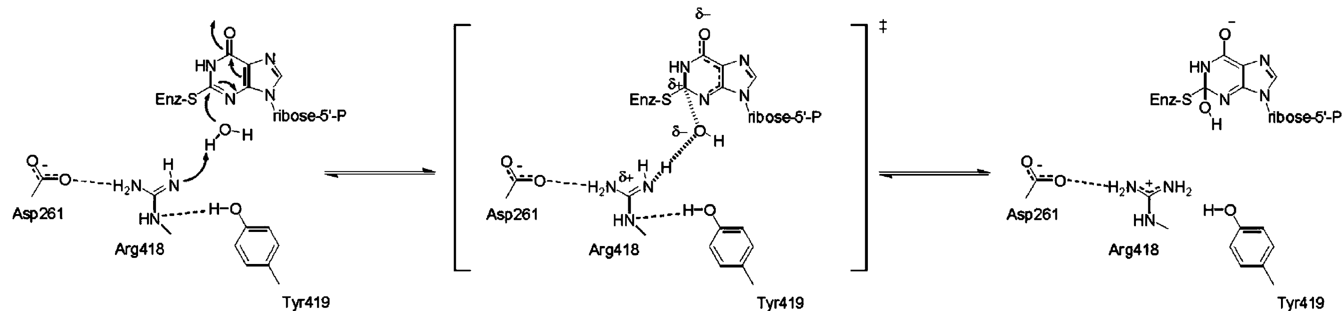
Tyr419 Is Not Required for Chemical Rescue. The Arg418Ala/Tyr419Phe double mutant was constructed to determine if Tyr419 is required for the rescue reaction. We characterized the pre-steady-state IMPDH reaction as described previously (*8*) and demonstrated that hydride transfer and NADH release have not been affected by the mutations. The rate constants for hydride transfer in the forward and reverse directions and NADH release are 52, 37, and 5.8 s^{-1}, respectively, as compared to wild-type values of 34, 59, and 8.5 s^{-1}, respectively (*8*). At present, we believe that the difference in the equilibrium constants for the hydride transfer reactions, 1.4 for the wild type versus 0.6 for the mutant, reflects the imprecision of the data. However, the proximal region of the flap contacts IMP and NAD^+, so it is possible that mutations of Arg418 and Tyr419 also have subtle effects on hydride transfer.

In contrast, no activity is observed for the Arg418Ala/Tyr419Phe variant under steady-state conditions ($k_{cat} < 2 \times 10^{-6}$ s^{-1}), indicating that the hydrolysis of E−XMP* is selectively disrupted by these mutations as expected. Aminoguanidine increases the activity of the double mutant by a factor of at least 1000 (Table 1), indicating Tyr419 is not required for the rescue reaction. These data further suggest that Tyr419 does not act as the general base that activates water.

Guanidine Derivatives Do Not Stabilize the Closed Conformation. Multiple-inhibitor experiments were performed with tiazofurin and ADP in the presence of guanidine derivatives to investigate the effect of the rescue agents on the conformational change (Table 2). The apparent values of K_i for tiazofurin and ADP do not change in the presence of a rescue agent, which suggests that the structure of the active site is intact. If the rescue agents stabilize the closed conformation, then the value of α will decrease with increasing concentrations of the rescue agent. However, the presence of the rescue agent does not change the value of α (Table 2). Therefore, the rescue agent does not shift the equilibrium toward the closed conformation but instead specifically rescues k_{HOH}.

Solvent Isotope Effects on the Rescue Reaction. The reaction of the Arg418Ala enzyme displays a solvent isotope effect (SIE) on V_{max} of 4.8 (*8*). A similar SIE of 4.2 is observed in the presence of 10 mM aminoguanidine ($V_{max\ rescue} = 0.036 \pm 0.011$) (Table 2). However, the SIE decreases with increasing concentrations of aminoguanidine, becoming ∼1 at 200 mM rescue agent (Table 2). This observation indicates that the rate-limiting step changes at high concentrations of aminoguanidine. Perhaps the structure of the flap is perturbed so that the closure of the flap becomes rate-limiting. This scenario predicts that the value of α will increase in the presence of the rescue agent. Unfortunately, the value of α is already close to 1 for the Arg418Ala

Scheme 3: Mechanism of Water Activation by Arg418 and Guanidine Derivatives



enzyme, so it would be difficult to detect such an increase. However, we do observe an increase in the value of α for the wild-type enzyme (to ~0.1 in the presence of 150 mM guanidine; Table 2), which is consistent with the hypothesis that high concentrations of the rescue agent perturb the structure of the flap. Similar V_{max} SIEs are also observed on the methyguanidine rescue reaction (Table 2).

pH Dependence of the Rescue Reaction. We also investigated the effect of pH on the rescue reaction. We chose conditions in the pH-independent region of the wild-type and mutant IMPDH reactions to avoid complications from other ionizations. The value of k_{rescue}/K_R increases with pH (Figure 2), as expected if the reaction involves a neutral guanidine rather than the guanidinium ion.

Bronsted Plot for the Rescue Reaction. We assessed the rescue reaction of two alkyl guanidines with pK_a's comparable to that of guanidine in an attempt to assess the steric contribution. In contrast to the examples cited above, a simple linear correlation is not observed between $\log(k_{rescue}/K_R)$ and the steric parameter E_s (Table 1). Instead, our results are consistent with a size exclusion model. The substitution of Ala for Arg will create a cavity that can accommodate methylguanidine, but a steric clash is expected between the Ala side chain and ethylguanidine. Similar results have been obtained for chemical rescue experiments in other systems (*20*−*23*).

We constructed a limited Bronsted plot using rescue agents that are expected to fit into the cavity. A reasonable

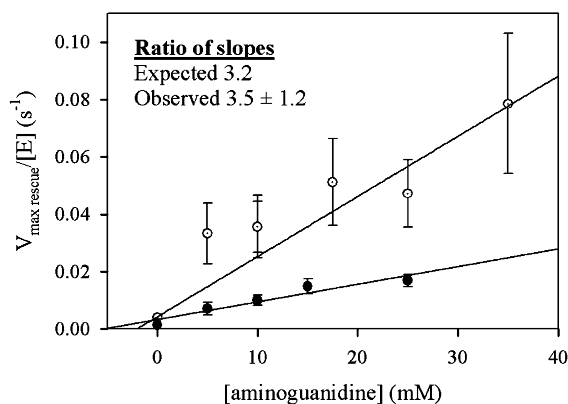


FIGURE 2: Effect of pH on k_{rescue}/K_R for the aminoguanidine-rescued Arg418Ala. The expected ratio of the slopes of the lines is based on a pK_a of 11 for aminoguanidine with the free base as the active species. The slope of the line is $(k_{rescue}/K_R)/(1 + [H^+]/K_a)$, and the intercept is the hydroxide-catalyzed reaction rate at the indicated pH. The filled circles show the dependence of the data at pH 7.5 and the empty circles that at pH 8. The data from the experiment at pH 8.0 were weighted by the reciprocal of the standard deviation squared.

correlation is observed between the rescue efficiency and the pK_a of the rescue agent, resulting in a Bronsted coefficient β of 1.1 ± 0.3 (R = 0.9), including hydroxyurea, and a β of 0.72 ± 0.3 (R = 0.85), when hydroxyurea is omitted (Figure 3). The failure to observe rescue with urea and thiourea is

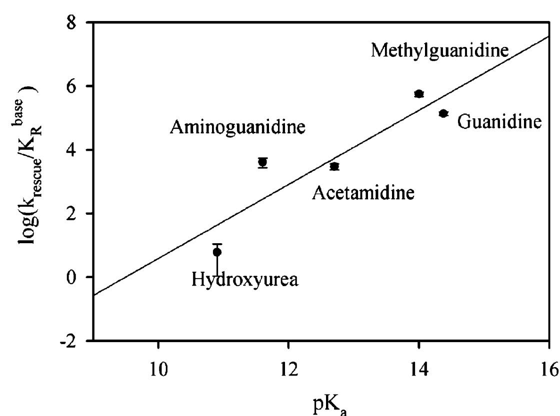


FIGURE 3: Bronsted plot of the rescue of Arg418Ala by guanidine derivatives. The Bronsted β values are 1.1 ± 0.3 (R = 0.9), including the hydroxyurea, and 0.7 ± 0.3 (R = 0.85), without hydroxyurea (not shown). Similar β values are obtained when the values of pK_a are normalized for the number of equivalent protons.

also consistent with this correlation. Of course, this is a small set of compounds, so the value of β cannot be considered well determined. We also recognize that others have shown that the binding properties of cavities can be difficult to predict (*20*).[2] Nevertheless, with these caveats, the Bronsted correlation suggests a late transition state where the proton has almost completely transferred to guanidine. This mechanism is consistent with the large normal SIE observed in the rescue reaction and predicts that the SIE will decrease as the rescue agent becomes more basic (*24*). Unfortunately, the SIEs do not have sufficient precision to address this question.

Implications for the Mechanism of IMPDH. The β value of ~1 suggests that the hydrolysis of E−XMP* has a late transition state where a proton has almost completely transferred from water to Arg418. However, the value of β does not provide information about the extent of bond formation between water and E−XMP*. We favor a mechanism involving concerted attack of water on E−XMP* with

[2] These experiments used alcohols to rescue His-to-Gly mutations; since alcohols are not expected to make the same network of interactions as a His, nonproductive binding modes could be more problematic. In contrast, in our experiments, the guanidinium group of the rescue agent can form the same network of interactions as Arg418, which would orient the rescue agents in the active site.

Arg418 acting as the general base (Scheme 3) because this mechanism can account for the large normal SIE observed in the rescue reaction. The β value of $\sim$1 could also be consistent with a stepwise reaction involving initial proton transfer to Arg418 followed by the attack of hydroxide on E−XMP*, but this mechanism is not consistent with the SIE [an inverse SIE is expected if the reaction involves hydroxide (*25*)]. A third alternative is a stepwise mechanism involving an initial attack of water on E−XMP* followed by proton transfer to Arg418. This mechanism is also unlikely, first because water is a poor nucleophile but also because proton transfer from the initial adduct should be fast, and thus cannot account for the SIE. Therefore, the mechanism of Scheme 3 is the simplest model that can account for the data.

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