Communications to the Editor

Rational Redesign of a Metal-Dependent Nuclease. Engineering the Active Site of Magnesium-Dependent Ribonuclease H To Form an Active "Metal-Independent" Enzyme

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Recent advances in the understanding of structure−activity correlations have fueled interest in the *de novo* design or modification of functional properties of enzymes by use of site-directed mutagenesis.[1−4] In this paper we demonstrate how a critical appraisal of molecular mechanism, in this case the role of an essential metal cofactor in enzyme catalysis, can provide the insight required for rational reconstruction of active site residues to produce a metal-independent enzyme exhibiting significant levels of activity. The enzyme selected for study was the magnesium-dependent endoribonuclease H, which hydrolytically cleaves the ribonucleotide backbone of RNA/DNA hybrids to produce 5′-phosphate and 3′-hydroxyl oligonucleotides.[5] The *Escherichia coli* enzyme is structurally homologous to the RNase H domain of HIV reverse transcriptase and shows retention of key active site residues.[6] Recent studies suggest that hydrolysis is promoted by one essential metal cofactor,[7−11] which is typically divalent magnesium. Intensive research efforts in our laboratory have resulted in the proposal of an unusual mechanism for enzyme-catalyzed metal-mediated hydrolysis of a nucleotide backbone: through stabilization of a transient intermediate by formation of an outer-sphere complex with the positively-charged metal cofactor.[12] This observation suggested to us the interesting hypothesis that mutation of active site carboxylate residues to positively-charged Lys or Arg might provide sufficient positive charge density and hydrogen-bonding propensity in the active site domain to mimic the role of hydrated divalent magnesium. In this paper we report the successful demonstration of this idea.

Table 1. Kinetic Parameters for Ribonuclease H Digestion of Hybrid Substrate

enzyme[a]	k_{cat} (substr s^{-1})[b]	enzyme[a]	k_{cat} (substr s^{-1})[b]
native	28 ± 8	Asp10Glu	14 ± 4
native (−Mg^{2+})	0	Asp10Asn/Ser/Arg/Gly	0
Glu48Asp[c]	1.1 ± 0.3	Asp10Arg/Asp70Lys	0
Glu48Gln[c]	0	Asp10Arg/Glu48Arg	24 ± 7[d]
Asp70Asn[c]	0	(−Mg^{2+})	

[a] Site-directed mutagenesis of the *rnh* gene in pET 21b(+) plasmid was carried out by Kunkel's method.[15] Mutants were screened by DNA sequencing following Sanger's chain termination method[16] and over-produced in *E. coli* BL21 (DE3). Protein purification protocols have been described elsewhere.[8] Purity was determined by SDS−PAGE followed by Coomassie Brilliant Blue staining. [b] Unless otherwise indicated, all reactions were carried out in 20 mM Tris base, pH 7.5, 50 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, saturating poly(A)-poly(dT), 10 mM M^{n+}, 570 nM RNase H, and 25 ± 1 °C. Data was obtained by use of an OLIS (On-Line Instrument Systems, Inc.) stopped-flow apparatus as described in detail elsewhere.[9] At least four data sets were averaged for each v_o. [c] Data adapted from ref 17. [d] For the Asp10Arg/Glu48Arg mutant the optimal activity obtained at pH ≈ 5.5 is reported. Product inhibition is observed at longer assay times.

These experiments also provide firm support for the mechanism of metal-mediated hydrolysis described in earlier work from our laboratory.[9,12,13]

Our design of the engineered active site was guided by recent crystallographic studies of the Mg^{2+}-bound enzyme.[7] Of the three principal active site carboxylate residues, crystallographic evidence suggests that both Glu48 and Asp10 are bound to Mg^{2+}, while Asp70 is proposed to serve as a catalytic base that is required for deprotonation of water prior to hydrolysis of the backbone.[7] We selected Asp10, Glu48, and Asp70 for mutational studies.[15,16] Mutation of the essential catalytic base (Asp70Asn) results in inactive enzyme.[17] Single-point mutations of the magnesium-binding residues, Asp10 and Glu48, yielded inactive enzyme either with or without added Mg^{2+} (Table 1), with the expected exception of the Asp10Glu and Glu48Asp mutants, which retained 50% and 4% activity, respectively. Introduction of a single positive charge (for example, Asp10Arg) is insufficient for activation, while also serving to inhibit binding of magnesium cofactors.[14] In contrast, at pH 5.5 the double mutant Asp10Arg/Glu48Arg demonstrated up to 87% of the maximum activity of the native enzyme, even in the absence of added Mg^{2+}. Substrate activation by native enzyme is promoted through transition state stabilization,[12,18] which is dominated by hydrogen bonding from waters of solvation. In the double mutant, the role of the hydrogen bond donor is accommodated by the guanidinium centers of the Arg side chains, since there is only a small increase in transition state energy (~0.1 kcal mol^{-1}) relative to the reaction promoted by hydrated magnesium.[9]

The pH dependence of activity for the Asp10Arg/Glu48Arg is shown in Figure 1 and is distinct from that of the native Mg^{2+}-promoted activity. Each can be fitted to yield similar ionization

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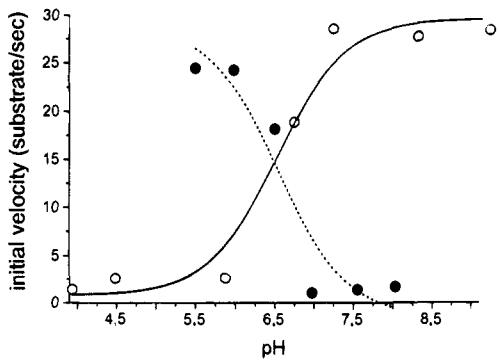

Figure 1. Variation of activity with pH for the Asp10Arg/Glu48Arg mutant (●). Data for native RNase H is shown for comparison (○).[19]

constants (pK_a's ≈ 6.5 and 6.6, respectively). Loss of activity at lower pH for the native enzyme reflects protonation of Asp10, a Mg^{2+}-binding residue.[19] In contrast, the activity of the Asp10Arg/Glu48Arg mutant is optimal at pH values immediately below the pK_a, and we ascribe the loss of activity at higher pH to deprotonation of one of the three basic residues in the active site of the mutant enzyme (Arg10, Arg48, and His124). A significant decrease in pK_a for arginine is possible, by analogy with the increase in pK_a observed for Asp10 in the native enzyme,[8,20] but this has not yet been established.

Two-dimensional NMR analysis of each mutant indicated minimal conformational change relative to the native protein (supporting information). Published crystallographic data obtained from the native enzyme with and without bound Mg^{2+},[21,22] and from active site mutants,[23] have demonstrated localized perturbations in side chain orientations and hydrogen-bonding patterns in the Mg^{2+}-binding pocket. However, the striking result reported herein, demonstrating up to 87% recovery of native activity by the double mutant in the absence of metal cofactor, does not support a role for these minor structural changes in catalytic activation.

These results demonstrate how a critical appraisal of molecular mechanism, in this case the role of an essential metal cofactor, can provide the insight required for rational engineering of an enzyme's active site. In contrast to previous studies,[1-4] the net enzymatic reaction has not been altered. Rather, the active site has been modified to provide the important catalytic components as an integral structural element of the enzyme's catalytic apparatus.

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Supporting Information Available: Aromatic and aliphatic regions of the 2D COSY spectra (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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