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GLU-416 of β-Galactosidase (*Escherichia coli*) Is a MG^{2+} Ligand and β-Galactosidases with Substitutions for GLU-416 Are Inactivated, Rather than Activated, by MG^{2+}

Nathan J. Roth, and Reuben E. Huber[1]

Division of Biochemistry, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

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Glu-416 of β-galactosidase (*E. coli*) was replaced with Gln and Val using site-directed mutagenesis. The substituted enzymes displayed a greatly decreased sensitivity to Mg^{2+}. Equilibrium dialysis studies indicated that wild-type β-galactosidase bound Mg^{2+} tightly, whereas E416V-β-galactosidase did not. In addition, the pH profile of E416V-β-galactosidase was unaffected by the presence or absence of 1 mM Mg^{2+}. Surprisingly, both substituted enzymes were inactivated, rather than activated, by Mg^{2+} but high amounts of Mg^{2+} were needed (1 mM). E416Q-β-Galactosidase was unstable when stored in the presence of Mg^{2+}. The substituted enzymes displayed a dramatically lowered affinity for the synthetic substrate, ONPG, and for IPTG (a substrate analog inhibitor) in both the presence and the absence of Mg^{2+}.

β-Galactosidase from *E. coli* requires Mg^{2+} for full catalytic activity and binds one Mg^{2+} per monomer (1). In the absence of Mg^{2+} only 6% of the catalytic activity is retained. The role of Mg^{2+} may be structural (2,3), although a direct catalytic role has also been suggested (4). The structure of β-galactosidase (5), determined to 3.5 Å, indicates that Glu-461, His-418, and Glu-416 are positioned in such a way that they could function as metal ligands at the active site. Mutagenesis studies of Glu-461 and His-418 have indicated that those residues are required for normal metal activation of β-galactosidase and are probably inner sphere ligands to Mg^{2+} (6–8).

The purpose of the studies reported here was to determine whether Glu-416 is also required for interaction with Mg^{2+}. Glu-416 was replaced by Gln and Val. Gln has a side chain that is approximately the same size as Glu, and contains an amido group capable of being a weak ligand to Mg^{2+} (9). Replacement of Glu with Val introduced an aliphatic group that is incapable of coordinating to a Mg^{2+} ion.

EXPERIMENTAL PROCEDURES

Chemicals and reagents. Enzymes used for site directed mutagenesis were purchased from Gibco BRL Life Technologies Inc. and DNA sequencing reagents were from Pharmacia Biotechnology Inc. or ICN Biomedicals Inc. All other chemicals were purchased from Sigma or similar sources.

Site-directed mutagenesis. All general molecular biology methods used were those of Sambrook *et al.* (10). The method utilized for site directed mutagenesis was a modified version of Kunkel's *dut- ung-* method (11). A DNA fragment of the *lacZ* gene containing the codon to be mutated was excised from the plasmid pIP 101 using Sst I and Cla I and ligated into the pBS SK+ vector (Stratagene) previously digested with the same endonucleases. The resultant subclone was transformed into competent *E. coli* RZ1032. ssDNA was isolated with the help of a helper phage (VCS M13) and this was used as the template DNA for mutagenesis. A phosphorylated primer containing the desired codon(s) was annealed to the ssDNA template. T4 DNA polymerase and T4 DNA ligase were added to the annealed primer-template mixture to synthesize and ligate the mismatched complementary DNA strand. The reaction mix was then transformed into *E. coli* XL1-Blue. Colonies were sequenced directly to confirm the mutation. Mutants were cloned back into the pIP expression vector using the restriction enzymes Sst I and Cla I. The integrity of the mutation in the final pIP mutant plasmid was reconfirmed by DNA sequencing.

[1] Fax: (403) 289-9311.

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; ONPG, o-nitrophenyl-β-D-galactopyranoside; TES, N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

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Enzyme purification. The wild type and substituted β-galactosidases were purified with only slight modifications to a previously described method (6). The β-galactosidase concentrations (mg/mL) were determined using an absorptivity coefficient of 2.09 cm^2/mg at 280 nm (12).

Enzyme assays. The k_{cat} and K_m values (25°C) for the wild type and mutant β-galactosidases were determined for ONPG in TES assay buffer (30 mM TES, 145 mM NaCl, pH 7.0 at 25°C) at 1 mM, 20 mM, and 50 mM $MgSO_4$ concentrations as well as in the absence of divalent metal ions (with 10 mM EDTA present). An extinction coefficient of 2650 $M^{-1}cm^{-1}$ was used for o-nitrophenol at pH 7.0. The values of k_{cat} and K_m were obtained from Eadie-Hofstee graphical analyses using the Enzyme Kinetics software program (Version 1.4) developed by Trinity Software.

Activity stains. Activity stains of native PAGE gels were performed using the procedure of Marchesi *et al.* (13).

Equilibrium dialysis. All buffers for equilibrium dialysis were made with Milli-Q water. The enzymes were passed through a Superose 6 column linked in series to a Superose 12 column. The system was pre-equilibrated with TES Assay Buffer containing 10 μM Mg^{2+} (30 mM TES, 145 mM NaCl, 10 μM $MgSO_4$, pH 7.00 at 25°C) and the enzymes that eluted were concentrated to 2 mg/mL using a Microsep centrifugal concentrator (cutoff 30 kDa). The enzyme solutions were placed in Spectra/Por cellulose dialysis tubing (cutoff 12–14 kDa) and dialyzed extensively against TES Assay Buffer containing 10 μM Mg^{2+}. After the final buffer change, the enzymes were allowed to dialyze for 24 h to ensure that equilibrium had been reached. An atomic absorption spectrophotometer (Perkin Elmer 5000) was standardized with a Mg^{2+} standard and the enzymes and final buffer dialysates were analyzed to determine the amount of Mg^{2+} present.

RESULTS AND DISCUSSION

The β-galactosidases with substitutions at Glu-416 were purified to homogeneity as viewed by SDS-PAGE (Figure 1A). E416V-β-Galactosidase was stable at 4°C for at least 4 months in TES assay buffer containing 1 mM Mg^{2+}. However, the E416Q-β-galactosidase lost activity during storage with Mg^{2+} (1 mM). Analysis of the ''inactivated'' pure E416Q-β-galactosidase preparation by SDS-PAGE (Figure 1B) revealed that only a small band remained that corresponded to the β-galactosidase monomer, whereas several new fragments of lower molecular weight became apparent. When this enzyme was stored in the presence of 10 mM EDTA, it retained its activity for at least 4 months, and still migrated as a single monomeric band on SDS-PAGE. Thus Mg^{2+}

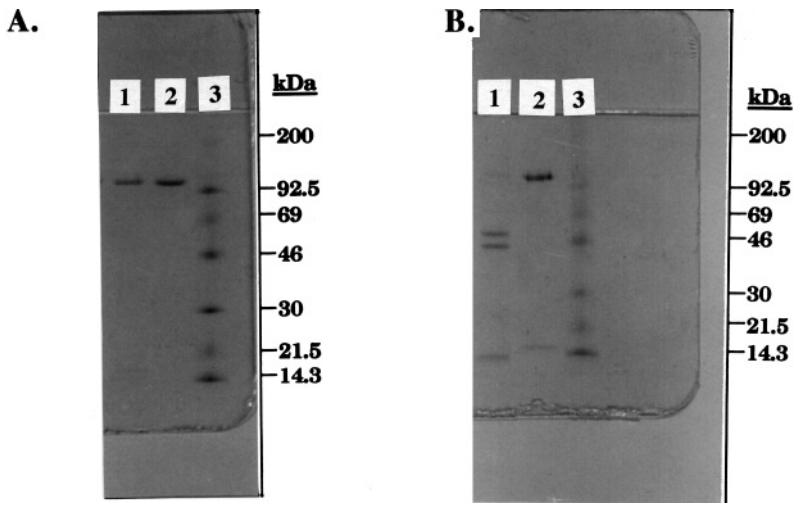

FIG. 1. SDS-PAGE of the β-galactosidases with substitutions for Glu-416. Approximately 0.5 μg of each enzyme was run on a 10–15% polyacrylamide gradient Phastgel. Samples were electrophoresed for 75 Vh at 15°C using Pharmacia's Phast system. The proteins were visualized by Coomassie blue staining. (A) Lane 1, E416Q-β-galactosidase stored in 10 mM EDTA; Lane 2, E416V-β-galactosidase stored in 10 mM EDTA; Lane 3, molecular weight markers (myosin (200,000), phosphorylase B (92,500), bovine serum albumin (69,0000), ovalbumin (46,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), and lysozyme (14,300)). (B) Lane 1, E416Q-β-galactosidase stored in 1 mM Mg^{2+}; Lane 2, E416V-β-galactosidase stored in 10 mM EDTA; Lane 3, molecular weight markers (as above). The storage stock of the E416Q-β-galactosidase in 1 mM Mg^{2+} (80 mM Tris, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM EDTA, 0.04% Na Azide, pH 7.5, at 4°C) was compared to a supply of the enzyme stored in the presence of 10 mM EDTA (30 mM TES, 145 mM NaCl, 10 mM EDTA, pH 7.0, at 25°C).

somehow renders E416Q-β-galactosidase susceptible to proteolytic degradation. This unexpected property of the enzyme limited the studies that could be done (in the presence of Mg^{2+}) to analyses that did not require large amounts of time. When prepared and stored in the absence of Mg^{2+}, both of the β-galactosidases substituted at Glu-416 eluted from the Superose 6/Superose 12 column in the same volume fractions as the wild type enzyme and migrated to a similar position as the native wild type enzyme when examined by non-denaturing PAGE (not shown). There was, however, a small amount of an extra band present with the non-denaturing PAGE of the E416V-β-galactosidase preparation. It appeared slightly above the tetramer band. Activity stains of native gels showed that both the tetramer band, and the extra band had β-galactosidase activity (data not shown). The intensities of the activity stained bands corresponded closely to the relative intensities of the Coomassie Blue stained bands and thus the small amount of protein in the slower migrating band was probably an aggregate that had full activity.

The effect of Mg^{2+} upon the kinetic constants of the enzymes was determined (Table I). As expected, wild type β-galactosidase (which has a dissociation constant of about 1 μM (1)) was fully activated in the presence of 1 mM Mg^{2+}. In the presence of 10 mM EDTA (very low free Mg^{2+}), the k_{cat} values of the E416Q-β-galactosidase and the wild type enzyme were actually similar. However, E416Q-β-galactosidase became inactivated (rather than activated) by the presence of high amounts of Mg^{2+}. The k_{cat} of metal-free E416V-β-galactosidase was significantly higher than that of the metal-free wild type enzyme, which may signify a conformation, in the absence of Mg^{2+}, that allows for better placement of functional groups in E416V-β-galactosidase. E416V-β-Galactosidase was also inactivated, rather than activated, when high concentrations of Mg^{2+} were present. The binding of Mg^{2+} by these enzymes with substitutions at position 416 must produce a change at the active site that is deleterious to activity. Previous studies (6) have shown that Glu-461 may play a direct role of some type in catalysis. It is possible that the total of the interactions with Mg^{2+} at the active site are such that enough negative charge is left on Glu-461 to allow direct interactions in catalysis. If Glu-416 (the subject of this report) is substituted by residues without negative charges, the complexing of Glu-461 to Mg^{2+} should be stronger (because one negative

TABLE 1
Kinetic Constants for the Glu-416 Substituted and Wild-Type Enzymes in the Presence of Various Mg^{2+} Concentrations with ONPG as Substrate[a]

		E416Q	E416V	Wild
[Mg^{2+}] (mM)				
0[b]	k_{cat}(s^{-1})	32	89	38
	K_m(mM)	6.4	5.4	0.57
	k_{cat}/K_m[d]	5.0	16.5	67
1	k_{cat}(s^{-1})	25	81	620
	K_m(mM)	13.4	6.5	0.12
	k_{cat}/K_m[d]	1.9	12.5	5200
20	k_{cat}(s^{-1})	1.7	18	580
	K_m(mM)	7.40	13.5	0.11
	k_{cat}/K_m[d]	0.23	1.3	5300
50	k_{cat}(s^{-1})	*c*	19.5	*c*
	K_m(mM)	*c*	23.3	*c*
	k_{cat}/K_m[d]	*c*	0.8	*c*

[a] The k_{cat} and K_m values were determined at 25°C in TES assay buffer (30 mM TES, 145 mM NaCl, and the appropriate concentration of $MgSO_4$, pH 7.00) with ONPG as the substrate.

[b] In the presence of 10 mM EDTA and no added Mg^{2+}.

[c] Not determined.

[d] (s^{-1}/mM^{-1}).

charge of the complex is lost) and the negative charge on Glu-461 should be decreased. This could be the reason that activity is lost when Mg^{2+} binds to β-galactosidases that have substitutions for Glu-416.

The amount of Mg^{2+} needed to affect the activity (Table I) was analyzed. It is reasonable to assume that the lowering of the k_{cat} and the k_{cat}/Km values that occurs upon increasing the concentration of Mg^{2+} is an indication of the stability of the interaction. The Mg^{2+} concentrations at which k_{cat} and k_{cat}/Km were at half of their maximal values should approximate the dissociation constants for Mg^{2+} binding. Analysis of the data on Table I indicates that both E416Q- and E416V-β-galactosidases have dissociation constants between 1 and 20 mM Mg^{2+}. This is significantly higher than the Mg^{2+} dissociation constant (about 1 μM) for wild type β-galactosidase. Close analysis of the data (see especially the decrease in the values of the k_{cat}/Km values as the Mg^{2+} concentration was increased), indicates that E416Q-β-galactosidase may bind Mg^{2+} a little better than does E416V-β-galactosidase. It is not surprising that an amide would retain somewhat more Mg^{2+} binding ability than would a hydrocarbon chain.

In the absence of divalent metals (10 mM EDTA), both of the substituted enzymes had K_m values (Table I) that were approximately an order of magnitude higher than the corresponding value for wild type. At higher levels of Mg^{2+}, the K_m values either increased or stayed at about the same value for both substituted β-galactosidases. In the case of the wild type enzyme, the K_m values decrease when Mg^{2+} is added. K_s values for β-galactosidases with any substrate are always equal to or higher than the K_m values (because $K_m = K_s[k_3/k_2 + k_3]$ (6)). In addition, it was found that E416V-β-Galactosidase displays a lower affinity (about 300 fold) for IPTG (K_i = 25 mM; I mM Mg^{2+}), as compared to wild type β-galactosidase (K_i = 0.085 mM; 1 mM Mg^{2+}). Therefore, substitutions of Glu-416 seem to have large deleterious effects upon the ability of these enzymes to bind substrate (and substrate analog inhibitors) and this indicates that Glu-416 and the Mg^{2+} binding site are intimately involved in structuring the substrate binding site. Similar results have been observed for β-galactosidases with substitutions at His-418 and Glu-461 (6,8), suggesting an intimate association between Mg^{2+} and its ligands and the substrate binding site.

Equilibrium dialysis of E416V-β-galactosidase was carried out in the presence of 10 μM Mg^{2+}. E416V-β-galactosidase bound only 0.15 Mg^{2+}/monomer. This small amount was probably due to non-specific binding and is similar to the amounts reported for other mutants of β-galactosidase that bind Mg^{2+} poorly (8) and for the basal level of Mg^{2+} binding by wild type β-galactosidase (1). The poor metal binding is in good agreement with the effect of Mg^{2+} on the kinetic properties of the enzyme (Table I). The binding of Mg^{2+} by E416Q-β-galactosidase was not determined because E416Q-β-galactosidase was not stable enough in the presence of Mg^{2+} to do an equilibrium dialysis experiment.

pH profiles of k_{cat} were obtained for wild type and E416V-β-galactosidase with 1 mM Mg^{2+} and with 10 mM EDTA (Figure 2). The profiles of the k_{cat} values for the wild type β-galactosidase were, of course, markedly different in the presence versus the absence of Mg^{2+}. On the other hand, the profiles of the E416V-β-galactosidase were essentially the same in the presence of 1 mM Mg^{2+} as they were in the absence of the metal (10 mM EDTA). The similarity of the pH profiles of E416V-β-galactosidase provides a further indication of the limited effect of 1 mM Mg^{2} and shows that E416V-β-galactosidase does not bind a significant amount of Mg^{2+} is present at 1 mM. The pH profiles were also quite different in shape from those of wild type β-galactosidase. Substitution of the other Mg^{2+} coordinating ligands, Glu-461 and His-418, also has profound effects upon the pH profiles (6,8). The k_{cat} values for the E416V-β-galactosidase could not be determined at higher pH values than shown on Figure 2B because the K_m values increased sharply with pH and vastly exceeded the limitations of substrate solubility. The pH profiles of E416Q-β-galactosidase could not be determined for the same reason. The Km values of E461Q-β-galactosidase increased very dramatically at pH values higher than 7.0 and accurate Km values could simply not be obtained.

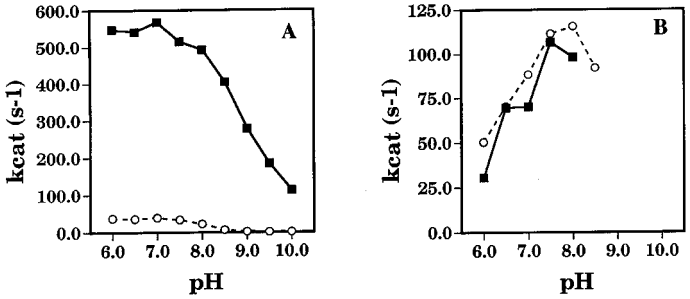

FIG. 2. pH profiles (ONPG) of the k_{cat} values of wild-type and E416V-β-galactosidase in the presence of 1 mM Mg^{2+} compared to 10 mM EDTA. The activities of the enzymes were determined at 25°C in pH assay buffer (30 mM TES, 50 mM histidine, 145 mM NaCl, pH adjusted at 25°C) in the presence of 1 mM $MgSO_4$ or 10 mM EDTA, with ONPG as the substrate. Each β-galactosidase variant is shown in a separate plot. The true k_{cat} values under each condition for each variant are plotted; 1 mM Mg^{2+} (■); 10 mM EDTA (&hb9;). (A) Wild-type β-galactosidase; (B) E416V-β-galactosidase.

It is interesting that β-galactosidases with substitutions for Glu-416 had very high Km values at high pH values. This was not observed (7,8) with β-galactosidases that had substitutions for Glu-461 and His-418 (the other ligands for Mg^{2+}). Obviously, Glu-416 is important for maintaining the integrity of the β-galactosidase binding site at pH values higher than 7.

The above results strongly suggest that the side chain of Glu-416 is a ligand to the active site Mg^{2+} of β-galactosidase. Replacement of Glu-416 by Gln or Val produces β-galactosidases that are quite insensitive to the presence or absence of the metal. It is not totally surprising that the E416V- and E416Q-β-galactosidases still maintained a modest ability to be affected by Mg^{2+} (and by implication to bind Mg^{2+} to a certain extent) at very high concentrations of the metal. The substitution of Glu removes only one of the three coordinating ligands.

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