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SITE SPECIFIC MUTANTS OF ß-GALACTOSIDASE SHOW THAT TYR-503 IS UNIMPORTANT IN Mg^{2+} BINDING BUT THAT GLU-461 IS VERY IMPORTANT AND MAY BE A LIGAND TO Mg^{2+}

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SUMMARY. The Mg^{2+} concentrations required for half maximal activity, the dissociation constants, and the free energies of binding for Mg^{2+} bound to wild type ß-galactosidase and several site specific mutants are reported. The mutants have one of the following substitutions: Glu-461 substituted with Asp, Gln, Gly, His, or Lys; or Tyr-503 substituted with Phe, His or Cys. Substitutions for Tyr-503 had little effect on the affinity of the enzyme for Mg^{2+}, implying that Tyr-503 is not involved in Mg^{2+} binding. Neutrally charged amino acids substituted for the negatively charged Glu-461 significantly decreased the affinity of the enzyme for Mg^{2+} and substitution of postitively charged amino acids at this position further decreased the affinity. On the other hand, substitution by Asp (negative charge) at position 461 had no effect on the binding. Thus, the negatively charged side chain of Glu-461 is important for divalent cation binding to ß-galactosidase.

A divalent metal ion, either Mg^{2+} or Mn^{2+}, is required for maximal activity of ß-galactosidase. No X-ray structure is available for this enzyme and it is not known which amino acids are involved in binding divalent cations. Magnetic resonance studies (1) suggested that the divalent metal ion is located 0.8-0.9 nm from the aglycone of a competitive inhibitor bound in the active site and there is evidence (2) that the divalent metal ion does not act as an electrophile in the catalytic mechanism. The Mg^{2+} binds noncooperatively with a reported dissociation constant of 0.67 µM (3). Although Ca^{2+} binds to ß-galactosidase competitively with Mg^{2+}, the Ca^{2+} binding is weaker and gives much less activation than Mg^{2+} or Mn^{2+} (4).

Site directed mutagenesis has been used to substitute a range of amino acids for Glu-461 and Tyr-503 and analysis of the properties of the substituted enzymes has established the importance of these amino acids in the action of ß-galactosidase (5, 6, 7). To determine if Glu-461

ABBREVIATIONS: ONPG, o-nitrophenyl-ß-D-galactopyranoside; PNPA, p-nitrophenyl-a-L-arabinopyranoside; TES, (N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid; E461D-, E461Q-, E461G-, E461H-, or E461K-ß-galactosidase, mutants in which Glu-461 has been substituted with Asp, Gln, Gly, His, or Lys, respectively; Y503F-, Y503H-, or Y503C-ß-galactosidase, mutants in which Tyr-503 has been substituted with Phe, His or Cys, respectively.

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or Tyr-503 are involved in the binding of divalent metal cations, we tested the interactions of Mg^{2+} with mutants at these positions. The data reported in this paper show that substitutions which change the charge at position 461 drastically affect the interactions.

Many of the proteins for which the structure of the Ca^{2+} binding sites have been characterized from crystal structures (8, 9) bind Mg^{2+} at the same sites. Sequence similarity to the nine amino acids which are common to both continuous "EF hand" (8, 10) and partially continuous "lock washer" (11) Ca^{2+} binding sites has been used (12, 13) to find putative Ca^{2+} and/or Mg^{2+} sites in proteins and is used in this paper to search for a possible divalent metal binding site of ß-galactosidase. However, discontinuous ligands to Ca^{2+} are also known from X-ray structures, either as completely discontin-uous binding sites in which all of the ligands to the metal are located on diverse places throughout the polypeptide chain (9) or in conjunction with the partially continuous "lock washer" sequence (11). It must also be recognized that a binding site which prefers Mg^{2+} over Ca^{2+} (14, 15) may have nitrogen ligands to the metal (not normally found in Ca^{2+} sites) and, also, a more rigid octahedral geometry which is less likely to accomodate a continuous binding sequence.

MATERIALS AND METHODS

Materials. The EDTA used was ACS Reagent from Sigma which the manufacturer states has ≤0.001% Ca and ≤5ppm Mg . ONPG and PNPA were from Sigma and other chemicals were from Fisher or similar sources. Concentrations of all compounds used were determined by weight. The ß-galactosidases were purified by a procedure similar to that of Brake et. al. (16). Wild type enzyme was obtained from *E. coli* ML-308 and the mutant enzymes in which Glu-461 was substituted with Asp, Gln, Gly, His, or Lys or in which Tyr-503 was substituted with Phe, Cys, or His were obtained from various *E. coli* strains described previously (5, 6, 17).
Dissociation Constants. The concentrations of Mg^{2+} required for half maximal activity and dissociation constants for Mg^{2+} were determined at 25°C in 30 mM TES buffer (pH 7.0) containing 145 mM NaCl and various concentrations of $MgSO_4$ and/or EDTA. The kinetic methods used for these determinations assume that modulation of the ß-galactosidase activity by Mg^{2+} results from binding of the metal to the enzyme. The instability constants of Sillen and Martell (18) were used to calculate free [Mg^{2+}]. The Mg^{2+} concentrations required for half maximal activity were determined with saturating amounts of ONPG present. Wild type ß-galactosidase retains a low activity in the absence of Mg^{2+} and some of the mutant ß-galactosidases are more active in the absence of Mg^{2+}. Thus, the kinetic method of Dixon and Webb (19) was modified to take this into account. In order to obtain dissociation constants for the free enzyme (K_D) it was necessary to work at very low substrate concentrations so that most of the enzyme is present as free enzyme rather than enzyme-substrate complex. The substrate PNPA was, therefore, used for the kinetic analysis from which K_D was calculated because the K_m for PNPA is much larger than the K_m for ONPG for wild type ß-galactosidase (20). Thus, PNPA concentrations which were at least 10 fold less than the K_m (see Table I) in the presence of Mg^{2+} (which was always smaller than the K_m in the absence of Mg^{2+}) could be used without rapid substrate depletion in the kinetic assays. It can be shown that by accounting for the velocity in the absence of Mg^{2+} and neglecting the small amount of enzyme-substrate complex present, one can obtain the following equation for the ratio (r) of enzyme bound to metal at constant and very low substrate concentration:
$r = [E{\cdot}M] / [E] = (v - v^o) / (v^{Mg} - v)$. The Mg^{2+} concentrations for v^o, v and v^{Mg} are zero, intermediate and saturating, respectively. It can be shown by theoretical treatment which accounts for the small amount of enzyme in the enzyme-substrate complex, that a more exact value for r can be calculated from a more complicated equation after use of the simple equation stated above to obtain a first approximation for r. However, at the low substrate concentrations used, the changes brought about by this iterative proceedure were negligible and are not incorported into the results reported. Hill plots were used to test for cooperativity and to calculate the dissociation constants of Mg^{2+} from the E·M complex. The enzyme was preincubated for at least 2 h at 25°C for each

concentration of Mg^{2+}, so that equilibrium could be established. In several cases complete reversibility of the Mg^{2+} binding was demonstrated, by increasing the Mg^{2+} concentration after the kinetic analysis and monitoring the restoration of complete maximal activity.
Test Sequence. The nine amino acid test sequence used to search for a possible divalent metal binding site was a consensus of the first 9 amino acids of calcium binding sites found in several proteins (8,9,10,11). Three of the amino acids in positions 1,3,5, and 9 (which are potential liganding positions) were required to be Asp, Asn, Gln, Glu, Ser, or Thr (two of these were further required to be Asp or Glu) and the amino acid at position 6 was required to be Gly.

RESULTS AND DISCUSSION

The concentrations of Mg^{2+} required to reach half maximal activity ($[Mg^{2+}]_{50\%}$) at saturating substrate concentrations are tabulated in the first column of Table I. If the ß-galactosidase mechanism contained only one enzyme-substrate complex, then $[Mg^{2+}]_{50\%}$ values would be the dissociation constants of Mg^{2+} from that enzyme-substrate complex. But because there are two enzyme-substrate complexes in the ß-galactosidase mechanism (21) and because the rate determining step is different for the various enzymes with the different substitutions (7) these values represent dissociation from different enzyme-substrate complexes, depending on the enzyme. However, these $[Mg^{2+}]_{50\%}$ values do provide an indication of the affinity of these enzymes for Mg^{2+}. They show that the ß-galactosidases which differ at position 461 have very different affinities for Mg^{2+} and fall into three distinct pairs: tight binding to wild type and E461D-ß-galactosidase, poorer binding to E461Q- and E461G-ß-galactosidases, and weak binding to E461H- and E461K-ß-galactosidases. Thus, the substitution of negatively charged Asp for negatively charged Glu-461 does not significantly affect $[Mg^{2+}]_{50\%}$. However, when a neutral

TABLE I

Concentrations of Mg^{2+} required for half maximal activity ($[Mg^{2+}]_{50\%}$) were determined at saturating (2mM) concentrations of ONPG as a substrate. Michaelis constants for PNPA are designated as "K_m^{Mg}" in the presence of Mg^{2+} and as "K_m " in the absence of Mg^{2+}. The dissociation constants for Mg^{2+} bound to the free enzyme (K_D) and the free energy of binding Mg^{2+} to the free enzyme (ΔG^o) were determined using PNPA as a substrate. The concentrations of PNPA used were 0.10 mM for both wild type ß-galactosidase and E461H-ß-galactosidase and 0.015 mM for both E461Q- and Y503F-ß-galactosidase All of the values were determined at 25° in pH 7.0 TES buffer (* indicates that values were not determined)

	$[Mg^{2+}]_{50\%}$ (µM)	K_m^{Mg} (mM)	K_m (mM)	K_D (µM)	ΔG^o (kJ/mol)
Wild Type	0.64	5.4	18	2.0	-33
E461D	0.80	*	*	*	*
E461Q	30	0.16	0.23	240	-21
E461G	20	*	*	*	*
E461H	280	2.2	10.8	3000	-14
E461K	170	*	*	*	*
Y503F	0.10	0.19	0.27	1.0	-34
Y503C	0.39	*	*	*	*
Y503H	1.9	*	*	*	*

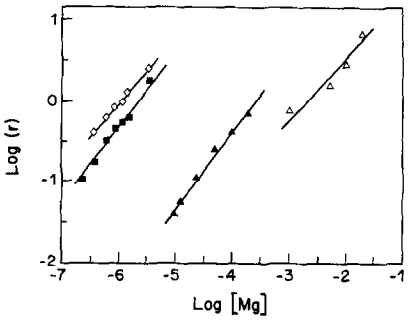

FIGURE 1. Hill Plot of the log(r) (where r=[E·M] / [E]) versus the log of the Mg^{2+} concentration at 25°C in TES buffer (pH 7.0). Unfilled diamond symbols are used for Y503F-ß-galactosidase, filled square symbols for wild type ß-galactosidase, filled triangular symbols for E461Q-ß-galactosidase, and unfilled triangular symbols for E461H-ß-galactosidase.

amino acid, Gln or Gly, is substituted for Glu-461 the $[Mg^{2+}]_{50\%}$ increases by about 30-fold and when a positively charged amino acid, Lys or His , is substituted for Glu-461 the $[Mg^{2+}]_{50\%}$ increases by about 300-fold. (Although the pKa of His-461 is unknown, it would be expected to be present at least partially in the protonated and thus positively charged form at pH 7.0 unless it is significantly perturbed.)

To establish that the differences were not due to dissociation from different enzyme-substrate complexes for the different enzymes, kinetic analysis of Mg^{2+} binding to the substrate free enzyme for one ß-galactosidase of each pair was carried out. The Hill plots obtained are shown in Figure 1 and the slopes are not significantly different from one. The dissociation constants for Mg^{2+}, which were obtained from the Hill plots, as well as the calculated free energies of binding are shown in the final two columns of Table I. The free enzymes all bind Mg^{2+} less well than do the enzyme-substrate complexes and the differences between the dissociation constants were even more dramatic (120-fold for E461Q and 1500-fold for E461H) than the differences between $[Mg^{2+}]_{50\%}$ values. Conformational differences caused by the disruptive effects of the substitutions may cause decreased binding in the mutants but the regular trend of decreasing affinity as the charge is increased and the very similar size of Gln and Glu are strong evidences that this is not the case.

The magnitude of the free energy of Mg^{2+} binding to free ß-galactosidase with the neutrally charged Gln at postion 461 is about 12 kJ/mol less than that for binding to wild type ß-galactosidase and the magnitude decreases by another 7 kJ/mol energy for binding to free ß-galactosidase with the positively charged His at position 461. Linse et. al. (22) have shown that removal of an individual negative charge decreases the magnitude of the free energy of binding of Ca^{2+} to calbinden up to 8 kJ/mol even though the charge is not part of the inner ligand sphere of the metal binding site. However, calculations with the dissociation constants of Marsden et. al. (23) show that removal of an individual negative charge from the inner sphere ligand decreases the magnitude of the free energy of binding of La^{3+} to a peptide analogue of the calcium-binding site of troponin C up to 12 kJ/mol. Since the change found here was about 12 kJ/mol, Glu-461 is probably an inner sphere ligand to the Mg^{2+} of ß-galactosidase. If it is not, it is clear that it must at

least be very close to the divalent metal binding site and that it significantly contributes to the binding.

The $[Mg^{2+}]_{50\%}$ values for the mutants at position 503 are also tabulated in Table I and indicate that all of them bind Mg^{2+} with roughly the same high affinity as the wild type. The dissociation constant of the substrate free enzyme for Y503F-ß-galacto-sidase confirms this. The fact that Y503F-ß-galactosidase actually binds Mg^{2+} a little more tightly than the wild type indicates that the hydroxyl of Try-503 is not a ligand to the divalent metal ion. Thus the rather large shift in the pKa of the alkaline group important for catalysis from 8.4 in the presence of Mg^{2+} to 6.5 in the absence of Mg^{2+} (3) can not be due to liganding of Mg^{2+} by Tyr-503.

Searching the ß-galactosidase primary structure yielded a possible divalent metal ion binding site at residues 15-23 with similarity to the continuous nine amino acid "lock washer"sequence found in galactose binding protein (11) and suggested in several other proteins (10,12). Thus the sequence Asp_{15}-Trp-Glu-Asn-Pro-Gly-Val-Thr-Gln_{23} may be suggested as a site for further study. Even though the results show that Glu-461 is important in Mg^{2+} binding, it is not included in the sequence above. Further searching which relaxed the requirement for two acidic amino acids in liganding positions or which accomodated the variation of the standard sequence found in the "pseudo EF hand" (10) yielded possible sequences near or even overlapping position 461 but none of them included Glu-461 in a liganding position. It is possible that the side chain of Glu-461 may act as a ligand to the metal if it is the discontinuous ligand not included in the continuous part of the "lock washer" type of binding site. It is also, of course, possible that the divalent metal binding site is a discontinuous site which can not be identified from sequence.

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