Articles

An Alkali Metal Ion Size-Dependent Switch in the Active Site Structure of Dialkylglycine Decarboxylase[†,‡]

Erhard Hohenester,[§] John W. Keller,[||] and Johan N. Jansonius*[,§]

Department of Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, and Department of Chemistry, University of Alaska, Fairbanks, Fairbanks, Alaska 99775

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ABSTRACT: The pyridoxal 5′-phosphate-dependent enzyme dialkylglycine decarboxylase (DGD) is activated by K^+ and Rb^+ ions, whereas Li^+ and Na^+ ions are inhibitory. A binding site for alkali metal ions close to the active site (site 1) was discovered in the crystal structure of DGD, and an exchange of K^+ for Na^+ at this site was shown to affect the conformation of two active site residues [Toney, M. D., Hohenester, E., Cowan, S. W., & Jansonius, J. N. (1993) *Science 261*, 756−759]. We have investigated the effects of alkali metal ions on DGD activity and have determined the crystal structures at 2.8 Å resolution of DGD with Li^+ and Rb^+ bound at site 1. Due to the weak scattering of the Li^+ ion, its position had to be modeled using information from small molecule structures. A comparison of the DGD structures with Li^+, Na^+, K^+, and Rb^+ bound at site 1 reveals a striking correlation between active site structure and enzymatic activity. The small, inhibitory ions Li^+ and Na^+ are accommodated by replacing two protein-derived ligands of the larger, activating ions K^+ and Rb^+ by a single water molecule. This actuates a two-state structural switch between active and inactive enzyme that involves a concerted reorientation of the active site residues Ser80 and Tyr301 and a small change in the quaternary structure of the DGD tetramer. An important role of the essential K^+ ion in both cofactor binding and the organization of a catalytically competent active site structure is proposed. In the structure of DGD with Rb^+ bound at site 1, a second Rb^+ ion has partially replaced the structural Na^+ ion at metal binding site 2 on the surface of the DGD molecule, without significantly altering the protein structure. In contrast to Na^+, the Rb^+ ion is bound with unfavorable geometry, and it is proposed that the rigid site 2 structure results in a pronounced selectivity for Na^+ ions.

Dialkylglyine decarboxylase (DGD,[1] EC 4.1.1.64) from *Pseudomonas cepacia* is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the degradation of small α,α-dialkylamino acids such as 2-aminoisobutyric acid (Aib). The catalytic mechanism of DGD is complex and involves two half-reactions (Bailey & Dempsey, 1967; Bailey *et al.*, 1970; Lamartiniere *et al.*, 1971; Keller *et al.*, 1990). In the first half-reaction, a dialkylamino acid is oxidatively decarboxylated to give CO_2 and the corresponding ketone, with amino group transfer to the cofactor PLP. The second half-reaction consists of a classical transamination reaction with pyruvate as the oxoacid substrate, yielding L-alanine. DGD has been shown to require K^+ ions for catalytic activity and stability, whereas Li^+ and Na^+ ions act as strong inhibitors of the enzyme (Aaslestad *et al.*, 1968). Until recently, the mechanism by which the different alkali metal ions exert these adverse effects on DGD activity has been obscure.

The crystal structure of DGD (Toney *et al.*, 1993) immediately offered a structural explanation for the activation and inhibition of DGD by alkali metal ions. DGD is composed of four identical subunits of 433 residues that form a loose dimer of intimate dimers. The active sites are located at the subunit interfaces of the functional intimate dimers. A metal binding site was discovered conspicuously close to the active site (site 1). In the presence of 15 mM K^+ and 75 mM Na^+, a K^+ ion with full occupancy is bound at this site (DGD-K^+; referred to as native A in Toney *et al.*, 1993), which is exchanged for a Na^+ ion, when the crystals are soaked in a solution containing 130 mM Na^+ as the only metal ion (DGD-Na^+; native B in Toney *et al.*, 1993). The exchange of ions at site 1 causes a notable rearrangement of two active site residues, Ser80 and Tyr301, which was proposed to account for the antagonistic effects of Na^+ and K^+ ions on DGD activity. The structural consequences of an ion replacement at site 1 extend far beyond the immediate

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[‡] The coordinates of the refined structures have been deposited under filenames 1DGD for the DGD-Li^+ structure and 1DGE for the DGD-Rb^+ structure with the Brookhaven Protein Data Bank, Brookhaven National Laboratory, Upton, NY, from which copies are available.

* Author to whom correspondence should be addressed.

[§] University of Basel.

[||] University of Alaska.

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[1] Abbreviations: DGD, dialkylglycine decarboxylase; DGD-M^+ (M^+ = Li^+, Na^+, K^+, Rb^+, Cs^+), DGD with M^+ bound at metal binding site 1; TPL, tyrosine phenol-lyase; PLP, pyridoxal 5′-phosphate; Aib, 2-aminoisobutyric acid; MES, 2-(*N*-morpholino)ethanesulfonic acid. A superscript asterisk (*) denotes residues that are contributed by the second, symmetry-related subunit in the dimer.

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vicinity of metal binding site 1. A detailed comparison of the DGD-Na^+ and DGD-K^+ structures revealed a small, but significant 1° rotation of the monomers with respect to each other in the dimer, *i.e.*, a small change in the quaternary structure of the DGD tetramer (Toney *et al.*, 1994).

In the DGD crystal structure, a second alkali metal ion binding site was found on the surface of the molecule (site 2), where a Na^+ ion is bound in a tight reverse turn at the carboxy terminus of an α-helix. This Na^+ ion was observed in both the DGD-Na^+ and the DGD-K^+ structures. While the K^+ ion at site 1 appears to be critical for producing and stabilizing a catalytically competent enzyme conformation, the Na^+ ion at site 2 is believed to play only a structural role.

The present paper describes an extension of our earlier work. We have investigated the effects of various mono- and divalent cations on DGD activity and have determined the crystal structures at 2.8 Å resolution of DGD with Li^+ and Rb^+ bound at site 1. Comparison of these new structures with the DGD-Na^+ and DGD-K^+ structures shows that the small, inhibitory cations Li^+ and Na^+ produce an active site structure that is different from the active site structure observed with the larger, activating cations K^+ and Rb^+, thus creating a metal ion size-dependent two-state structural switch between inactive and active DGD.

A vast number of protein structures with divalent metal ions (mainly Mg^{2+}, Ca^{2+}, and Zn^{2+}) bound are available (Glusker, 1991), whereas the interaction of proteins with alkali metal ions has been crystallographically documented only in very few cases. These include rhodanese which has monovalent cation binding sites of unknown function (Lijk *et al.*, 1984; Kooystra *et al.*, 1988), several serine protease structures where alkali metal ions are bound to low-affinity Ca^{2+} sites in the absence of high Ca^{2+} concentrations (Pantoliano *et al.*, 1988; Gros *et al.*, 1989), and cubic insulin crystals, which contain partially disordered binding sites for monovalent cations (Gursky *et al.*, 1992; Badger *et al.*, 1994). In none of these structures do the protein-bound alkali metal ions serve crucial structural or functional roles. On the other hand, many enzymes are known to absolutely require alkali metal ions for activity (Suelter, 1970), but the structural basis of monovalent cation activation in these enzymes is not understood. The alkali metal ion size-dependent structural switch in DGD offers a detailed view on how alkali metal ions can exert functional effects not less complex than those of divalent metal ions.

MATERIALS AND METHODS

Chemicals. DGD was purified as described (Keller *et al.*, 1990) and was stored at 4 °C in saturated ammonium sulfate solution. Metal-free enzyme[2] was prepared by replacing the storage solution with 30 mM triethanolamine/HCl, pH 7.9, 50 μM PLP using a Centricon-30 filtration device at 4 °C, with at least eight 2 mL changes of buffer ($>10^8$-fold dilution). Enzyme thus prepared did not show any appreciable activity under standard assay conditions in the absence of added KCl and was assumed to be metal-free, although metal analyses were not carried out. The specific activity of metal-free DGD after reactivation with 0.1 M KCl is diminished during storage of the enzyme in metal-free buffer. Therefore, only freshly prepared metal-free DGD was used for the metal ion activation experiments. Protein concentrations were determined spectrophotometrically, taking $E_{278} = 0.662$ for a 1 mg/mL solution (Lamartiniere *et al.*, 1971). α-[1-^{14}C]Aib was purchased from NEN Research Products; all other reagents were of analytical grade and were obtained from commercial sources.

Enzyme Assay. DGD activity was measured radiochemically by trapping $^{14}CO_2$ released from α-[1-^{14}C]Aib essentially as described (Keller *et al.*, 1990). Assays contained 1−5 μg of enzyme, 50 mM Aib (10 nCi/μmol), 5 mM triethanolamine/pyruvate, 50 μM PLP, 30 mM triethanolamine/HCl, pH 7.9, and varying amounts of metal chlorides in a total volume of 0.5 mL. All assays were performed at room temperature (20 °C). The enzyme was allowed to equilibrate with the respective metal chloride solution for 12 to 15 h at 4 °C before the reaction was started by the addition of substrates. CO_2 production was linear with time until ≈50% of the pyruvate was consumed. Activities were determined at <20% completion of the reaction. The overall yield of trapped CO_2 was determined in a substrate exhaustion experiment by adding a large excess of enzyme and is approximately 50%. Under the conditions employed, the specific activity in the presence of 0.1 M KCl was 3−5 μmol of CO_2/(min·mg), ≈200 times higher than the lowest specific activity that can be detected with this method. Control experiments with heat-inactivated enzyme did not detect a significant level of nonenzymatic Aib decarboxylation.

Crystallographic Analysis. DGD crystals were obtained as described (Toney *et al.*, 1991). DGD-Li^+ and DGD-Rb^+ crystals were prepared by soaking crystals grown in the presence of 15 mM K^+ and 75 mM Na^+ (DGD-K^+, referred to as native A in Toney *et al.*, 1993; typical size 0.4 × 0.4 × 0.7 mm^3) in solutions containing 20% PEG4000 (w/v), 100 mM pyruvic acid, 30 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) and 50 μM PLP, with either LiOH or RbOH added to give a final pH of 6.2 (approximately 130 mM metal ion concentration). Diffraction data were collected at room temperature on an Enraf-Nonius FAST area detector diffractometer, using Cu Kα radiation produced by an FR571 rotating anode generator (40 kV, 50 mA). The space group of the crystals is $P6_422$, with unit cell constants $a = b = 152.7$ Å and $c = 86.6$ Å for the DGD-Li^+ crystal and $a = b = 153.5$ Å and $c = 86.6$ Å for the DGD-Rb^+ crystal. Data were collected with an oscillation range of 0.15° per 120 s exposure and were processed with the program package MADNES (Messerschmidt & Pflugrath, 1987). The CCP4 program ROTAVATA/AGROVATA was used for scaling and merging of the data. Structure factor amplitudes were calculated with the CCP4 program TRUNCATE. Data collection and reduction statistics are summarized in Table 1.

Scaling of the DGD-Li^+ and DGD-Rb^+ data to the existing DGD-Na^+ (native B in Toney *et al.*, 1993) and DGD-K^+ data was performed with the CCP4 program SCALEIT, using all data in the 8 to 2.8 Å range. From the scaling *R*-factors so obtained (Table 1), the DGD-Na^+ and DGD-K^+ structures were judged to be suitable starting models for refinement of the DGD-Li^+ and DGD-Rb^+ structure, respectively. Initial difference Fourier maps showed pronounced differences at

[2] In this paper, the term "metal-free enzyme" is used rather loosely to describe catalytically inactive DGD most likely devoid of a metal ion at site 1. It is not known to which extent the structural Na^+ ion at site 2 is removed from the protein under the conditions employed to remove the cation bound at site 1.

Table 1: X-ray Data Collection and Refinement

parameter	structure DGD-Li^+	DGD-Rb^+
data collection		
resolution limit (Å)	2.8	2.8
no. of observations	80899	62340
no. of unique reflections	14859	14856
R_{merge} (%)[a]	10.0	10.8
completeness (%)	98.2	97.7
ΔF with DGD-Na^+ data (%)[b]	12.9	22.4
ΔF with DGD-K^+ data (%)[b]	17.9	11.4
refinement		
resolution range (Å)	8 to 2.8	8 to 2.8
no. of observations	14224	14232
protein atoms	3179	3206
metal ions	1 Na^+	2 Rb^+
water molecules	99	96
buffer molecules (MES)	1	1
R-factor (%)[c]	17.8	19.5
rms bond lengths (Å)	0.012	0.013
rms bond angles (deg)	2.6	2.4
rms planes (Å)	0.009	0.008
rms ΔB for bonded atoms (Å^2)	1.7	2.1

[a] $R_{merge} = \sum_{hkl}\sum_i|I(hkl)_i - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I(hkl)_i$, where $\langle I(hkl)\rangle$ is the average of $I(hkl)_i$ over all symmetry equivalents. [b] $\Delta F = \sum_{hkl}|F_{deriv}(hkl) - F_{native}(hkl)|/\sum_{hkl}F_{native}(hkl)$. [c] R-factor $= \sum_{hkl}|F_o(hkl) - F_c(hkl)|/\sum_{hkl}F_o(hkl)$.

metal binding site 1 for both DGD-Li^+ and DGD-Rb^+, in agreement with the expected exchange of ions. In addition, strong positive density at site 2 in DGD-Rb^+ indicated binding of Rb^+ to this site as well.

All metal ions and water molecules were removed from the starting models, and one Rb^+ ion each was introduced into the DGD-Rb^+ model at both metal site 1 and 2. Refinement of the DGD-Li^+ and the DGD-Rb^+ structure was then carried out with the program TNT (Tronrud *et al.*, 1987) against all observed data in the 8 to 2.8 Å range, with tightly restrained atomic temperature factors. Metal−ligand distances were not restrained in the refinement, and the van der Waals radii of the metals were set to zero. This procedure guarantees that the refined metal−ligand distances are not subject to any bias.

After partial refinement of the DGD-Rb^+ structure, the B-factor of the Rb^+ ion at site 2 was 73 Å^2. This value is much higher than the average B-factor of the protein atoms in this region (≈20 Å^2), indicating less than full occupancy of the Rb^+ ion. Several refinement trials with metal ion occupancies ranging from 0.2 to 0.8 showed that an occupancy of 0.5 produced a refined metal ion B-factor close to the values of its ligand residues, and a Rb^+ occupancy of 0.5 at site 2 was consequently used in the last refinement cycles. No attempts were made to simultaneously refine metal ion occupancy and B-factor, since these parameters are known to be highly correlated at medium resolution. The Rb^+ ion at site 1 refined with unit occupancy has a B-factor of 25 Å^2, compared to an average B-factor of its ligand atoms of 22 Å^2.

The refinement of the DGD-Li^+ structure was complicated by the fact that the Li^+ ion could not be located in electron density maps due to its low atomic number. Various models were constructed to account for the observed electron density at metal binding site 1. Refinement trials of these models, together with the use of information on the preferred coordination geometries of Li^+ in small molecule structures, resulted in the final DGD-Li^+ model. Since the interpretation of the electron density at site 1 in the DGD-Li^+ structure is central to this paper, the refinement of the DGD-Li^+ structure is described in detail below (see Results).

Table 2: Metal Ion Activation and Inhibition of DGD

cation	ionic radius[a] (Å)	relative activity[b] (%) 100 mM cation chloride	25 mM KCl + 100 mM cation chloride
none		<[c]	100
Li^+	0.60	<	56
Na^+	0.95	2	80
K^+	1.33	100	nd[d]
Rb^+	1.48	72	nd
Cs^+	1.69	5	77
NH_4^+	1.44	54	nd
Mg^{2+}	0.65	<	44
Ca^{2+}	0.99	<	22
Ba^{2+}	1.35	<	56

[a] Taken from Glusker (1991). [b] Assays contained 50 mM Aib, 5 mM triethanolamine/pyruvate, 50 μM PLP, 30 mM triethanolamine/HCl, pH 7.9, and metal chlorides as indicated and were carried out at 20 °C. Activity values are averages of at least three measurements. Estimated relative standard errors are 5−10%. [c] Catalytic activity below the detection limit of the assay (≈0.5% of the activity with 0.1 M KCl). [d] nd, experiment not done.

A total of 99 and 96 well-ordered water molecules were observed and refined in the DGD-Li^+ and DGD-Rb^+ structures, respectively. Both structures have a MES buffer molecule bound in the active site, as already described for the DGD-Na^+ and DGD-K^+ structures (Toney *et al.*, 1993). Refinement statistics for both DGD-Li^+ and DGD-Rb^+ are summarized in Table 1.

RESULTS

Metal Ion Activation Experiments. Metal-free DGD was prepared and its activation by various mono- and divalent cations was studied at essentially saturating concentrations of both substrates [the Aib and pyruvate concentrations in the assay correspond to 6 and 30 times their respective K_M values determined in the presence of K^+ ions (Lamartiniere *et al.*, 1971)]. At the PLP concentration used in the standard assay (50 μM PLP), the catalytic activity of the metal-free enzyme is <0.5% of the activity observed in the presence of 0.1 M KCl. At higher PLP concentrations, however, DGD is enzymatically active even in the absence of added activating cations. At 1 mM PLP, the activity of metal-free DGD is about 30% of the maximum activity in the presence of K^+ ions (not shown).

The data in Table 2 show that, of all cations tested, only K^+, Rb^+, and NH_4^+ are able to significantly activate DGD at low cofactor concentrations, Rb^+ and NH_4^+ being somewhat less effective than K^+. Na^+ and Cs^+ activate DGD to only a minor extent, whereas Li^+ and the alkaline earth metals are totally ineffective. Increasing the concentration of nonactivating or weakly activating cations above 0.1 M does not result in an increased catalytic activity (not shown). This indicates that the inability of these cations to activate DGD is not simply a result of a subsaturating ion concentration in the assay mixture (which was 0.1 M metal chloride). In addition, all nonactivating cations are inhibitory when added to assay solutions containing K^+ ions (Table 2), suggesting that nonactivating cations are competing with activating cations for binding to DGD.

It is concluded that, in the absence of very high (>1 mM) cofactor concentrations, DGD requires a monovalent cation

with an ionic radius of 1.3 to 1.5 Å for full enzymatic activity. A quantitative analysis of the effects of metal ions on the activity of DGD was not feasible within the scope of this work, and the qualitative observations reported here will be only used to aid in the interpretation of the crystalstructures of DGD with various alkali metal ions bound to the active site (see below).

Crystal Structure of DGD-Li$^+$. A DGD crystal, grown in the presence of 15 mM K^+ and 75 mM Na^+ and known to contain a K^+ ion with essentially full occupancy at site 1 (Toney *et al.*, 1993) was soaked for 24 h in a solution containing 130 mM Li^+ as the only metal ion. Diffraction data to 2.8 Å resolution were collected from this crystal and an F_o(DGD-Li^+) − F_o(DGD-K^+) difference Fourier map was calculated. As anticipated from the high scaling *R*-factor between the two data sets (Table 1), the resulting map was rather noisy. The most prominent features were observed in the immediate vicinity of metal binding site 1. Strong negative difference density (nine standard deviations below the mean) was observed at the position of the K^+ ion. In addition, a reorientation of the side chains of residues Ser80 and Tyr301 was evident from the difference Fourier map.

The DGD-Li^+ structure was refined after the metal ion at site 1 and all water molecules had been omitted from the model. Upon extensive refinement and the introduction of 97 well-ordered water molecules at positions distant from metal binding site 1, the *R*-factor converged at 0.179. At this stage, the electron density in the active site unequivocally showed that both the Ser80 and Tyr301 side chains had reoriented upon soaking (Figure 1A). In DGD-Li^+, the Ser80 hydroxyl group is pointing away from the metal ion and makes a hydrogen bond to the active site residue Gln52* of the neighboring subunit in the dimer,[3] thereby replacing the Tyr301 phenolic hydroxyl group as a hydrogen bond donor to Gln52*. Having lost its hydrogen bonding partner, the Tyr301 aromatic side chain is considerably disordered in DGD-Li^+ (as in DGD-Na^+).

An $F_o - F_c$ difference Fourier map, calculated with phases from this intermediate model, shows two spherical electron density features of similar height in the region around metal binding site 1 (Figure 1B). The "upper" electron density peak (in the view of Figure 1B) exactly coincides with the location of a water molecule that is observed in the DGD structures with Na^+, K^+, and Rb^+ bound at site 1 (see below). This metal ligand is found at an identical position in three structures with different alkali metal ions and coordination geometries. It is therefore very likely that an equivalent water molecule is present in the DGD-Li^+ structure. Consequently, a water molecule was added to the model at the position of the "upper" difference density peak and allowed to refine with full occupancy. The *B*-factor of this new water molecule after refinement was 35 Å^2, comparable to the average *B*-factor of the other 97 well-ordered water molecules (33 Å^2).

The second, "lower", difference electron density peak is observed at a position that is not occupied by either a metal ion or a water molecule in the other site 1 structures, although its maximum is close (≈0.7 Å) to the position of the K^+ ion in the DGD-K^+ structure. The closest distances of the peak maximum to surrounding protein atoms are 2.89 Å to the carboxylate oxygen atom $O_{\delta 1}$ of residue Asp307, and 2.85 Å, 2.87 Å, and 2.83 Å to the carbonyl oxygen atoms of residues Leu78, Thr303, and Val305, respectively. The distance to the upper water molecule described above is 3.18 Å. These distances are compatible with the lower difference electron density peak being either a water molecule or an alkali metal ion of at least the size of K^+. Both possibilities were tested. Refinement of a water molecule and a K^+ ion with full occupancy resulted in atomic *B*-factors of 29 Å^2 and >100 Å^2, respectively. Refinement of a K^+ ion with a fixed *B*-factor of 25 Å^2 (*i.e.*, the average *B*-factor of the protein atoms) resulted in an ion occupancy of 0.47. A K^+ ion with half occupancy at site 1 would imply that the DGD-Li^+ structure actually consists of a superposition of 50% of the parent DGD-K^+ structure (with a considerably different K^+ position, however) and 50% of an, as yet not defined, structure with a different metal binding site 1 and active site structure. This is highly unlikely for the following reasons: (1) The overall protein structure of DGD-Li^+ is significantly different from the DGD-K^+ structure (see below), yet the electron density for the polypeptide chain in the DGD-Li^+ structure is very well defined throughout the entire molecule. (2) The refined *B*-factors of the DGD-Li^+ structure are very similar to those of the DGD-K^+ and DGD-Rb^+ structures, which contain a metal ion with essentially full occupancy at site 1 (see below). (3) The final difference electron density map in the active site of DGD-Li^+ is featureless, especially around Ser80 and the position in DGD-K^+ of the Tyr301 side chain. These observations strongly argue against a superposition of structures, where one would expect diffuse electron density, high *B*-factors, and residual difference electron density in those regions where the two structures differ significantly. In conclusion, the two difference electron density peaks in Figure 1B are best interpreted as bound water molecules. Their inclusion into the crystallographic model lowered the *R*-factor to 0.178 and completed the DGD-Li^+ model. As shown in Figure 1C, both water molecules assigned at site 1 in DGD-Li^+ make favorable hydrogen-bonding interactions with the protein molecule.

The remaining question about the nature of metal binding site 1 in DGD-Li^+ concerns the presence or absence of a bound Li^+ ion. Since a Li^+ ion contains only two electrons, it is hardly surprising that the ion could not be located in difference Fourier maps (see also below). Instead, evidence from other sources had to be used to help settle this question. The presence of an "empty" metal binding site (*i.e.*, with no metal ion bound) can, in our opinion, be ruled out on the basis of the following observations: (1) All attempts to prepare metal-free DGD in the crystalline state by soaking crystals in solutions containing large organic cations yielded only disordered and poorly diffracting crystals, indicating that the removal of the ion at site 1 results in a gross structural change that essentially destroys the crystals. The DGD-Li^+ crystal investigated diffracted to 2.8 Å resolution and did not show a higher degree of disorder than its parent DGD-K^+ crystals. The much decreased stability of metal-free DGD with respect to enzyme saturated with K^+ (see Materials and Methods) also suggests a structural change upon metal ion removal. (2) Preliminary experiments indicate a dissociation constant of the Li^+ ion of about 5

[3] Based on the published primary structure of DGD (Keller *et al.*, 1990), residue 52 was described as glutamic acid in the first report on the DGD structure (Toney *et al.*, 1993). A reanalysis of the nucleotide sequence has shown that this residue is glutamine instead of glutamic acid (Toney *et al.*, 1994).

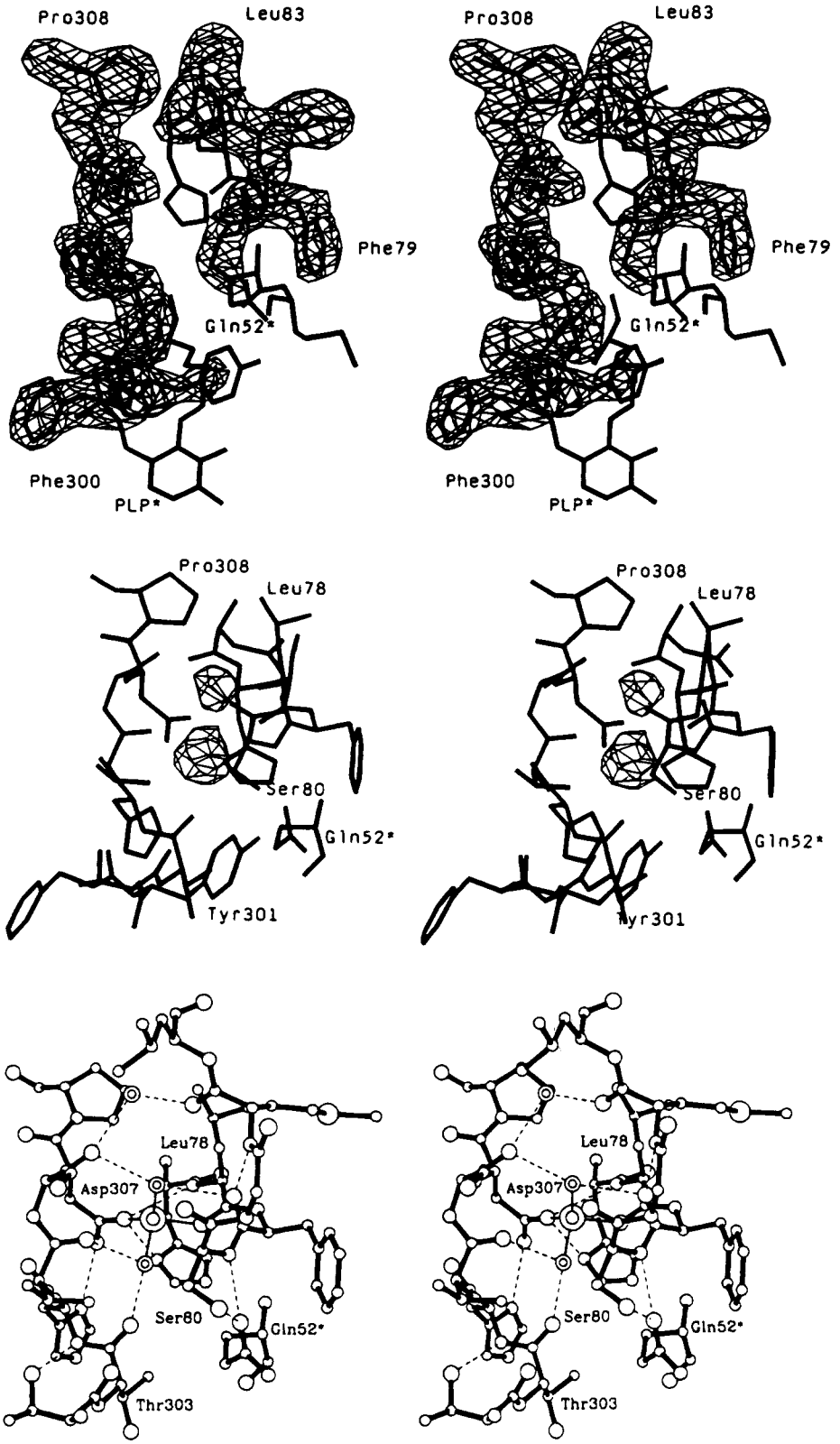

FIGURE 1: Structure of metal binding site 1 in DGD-Li^+: (A, top) Stereoview of an omit map around the metal binding site, calculated with $F_o - F_c$ coefficients and contoured at a level corresponding to 4 σ (standard deviations above the mean). Residues 79 through 83 and 300 through 308 were removed from the phasing model. The side-chain conformation of Ser80 is well defined by the electron density, whereas the side chain of Tyr301 is considerably disordered around a position different from the one it occupies in DGD-K^+ (see text). (B, middle) Stereoview of an $F_o - F_c$, α_c difference electron density map, calculated before the water molecules at site 1 were added to the crystallographic model and contoured at a level corresponding to 4 σ. The upper and the lower spherical density features have peak heights corresponding to 6σ and 8σ, respectively, and were interpreted as bound water molecules. (C, bottom) Stereoview of the modeled site 1 structure with Li^+ bound. The metal ion and water molecules are represented by a large and by small double circles, respectively. The Li^+ ion is tetrahedrally coordinated by the carbonyl oxygen atom of Leu78, the carboxylate oxygen $O_{\delta 1}$ of Asp307, and the two water molecules defined by the electron density. The average metal−ligand distance is 1.9 Å. Hydrogen bonds are represented by broken lines. Residue Gln52* is contributed by the other subunit in the dimer.

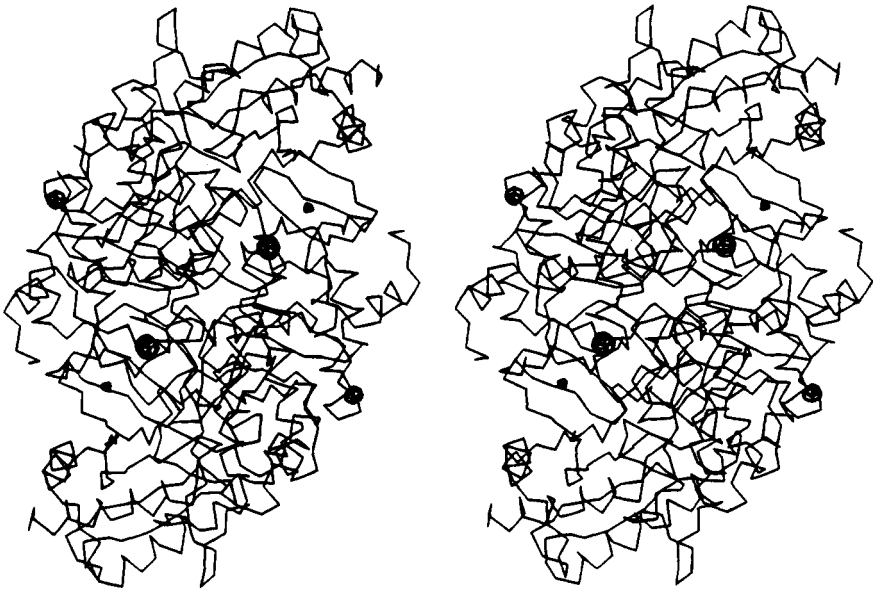

FIGURE 2: Stereoview of an α-carbon trace of the DGD dimer viewed down the 2-fold axis together with the F_o(DGD-Rb^+) − F_o(DGD-K^+), α_c(DGD-K^+) map contoured at a level corresponding to six standard deviations above the mean. The two spherical peaks in each subunit at the positions of metal binding sites 1 (internal) and 2 (on the surface) indicate binding of Rb^+ to each of the sites.

mM (E. Hohenester and J. N. Jansonius, unpublished results), and 130 mM Li^+ should therefore be more than sufficient to ensure a full Li^+ occupancy at site 1. Since there is no indication for a very low rate of the ion exchange at site 1, which would invalidate this argument, it is reasonable to assume that a Li^+ ion is present at site 1 in the DGD-Li^+ structure.

Lacking direct crystallographic observation of the Li^+ ion, information from small molecule structures was used to construct a model for the structure of site 1 with Li^+ bound. Typical Li^+−oxygen distances in small molecule structures are 1.9 to 2.0 Å (Shannon, 1976; Brown, 1988), with an average coordination number of the Li^+ ion of 4.9 (Glusker, 1991). In carboxylate salts, the Li^+ ion is often tetrahedrally coordinated by oxygen atoms (*e.g.* He & Craven, 1984) and strongly prefers to be in the plane of the carboxylate group (Carrell *et al.*, 1988).

Since the electrostatic interaction of the alkali metal ion with the Asp307 carboxylate group is conserved in all other site 1 structures, the Li^+ ion was placed in the carboxylate plane, such that it is coordinated by $O_{\delta 1}$ of Asp307, the carbonyl oxygen of Leu78, and by the two water molecules defined by the electron density (Figure 1C). The resulting coordination geometry is a remarkably regular tetrahedron, with an average metal−ligand distance of 1.9 Å. An alternative Li^+ position, with the Val305 carbonyl oxygen coordinating to the metal ion (as in DGD-Na^+), results in a more distorted coordination geometry and would place the Li^+ ion in a very unfavorable position with respect to the Asp307 carboxylate group (Carrell *et al.*, 1988).

The final model for the site 1 structure in DGD-Li^+, as presented in Figure 1C, is consistent with all available biochemical and structural evidence. More importantly, conceivable alternative models, such as a superposition of two structures, a metal-free site 1 structure, or a different Li^+ coordination, would be very difficult to reconcile with experimental observations.

Finally, we tested the validity of our assumption that the contribution of a Li^+ ion to the overall scattering from a protein of 433 residues is negligible at the current level of resolution. This was done by computing an $F_o - F_c$ difference Fourier map, with an arbitrarily placed Li^+ ion included into the structure factor calculation (with full occupancy and an atomic *B*-factor of 25 Å^2). If a Li^+ ion contributed significantly to the scattering, negative difference density should be observed at its position. This was not the case, even when a very low contouring level corresponding to only two standard deviations of the map was used.

The difference electron density in DGD-Li^+ at metal binding site 2 did not indicate any binding of Li^+ to this site, and a Na^+ ion with unit occupancy was therefore refined at site 2, as in DGD-K^+ and DGD-Na^+. The refined *B*-factor of this Na^+ ion in DGD-Li^+ is 19 Å^2. It thus appears that soaking in a solution containing 130 mM Li^+ as the only metal ion for one day did not remove the structural Na^+ ion at site 2.

Crystal Structure of DGD-Rb^+. A DGD-K^+ crystal was soaked in a solution containing 130 mM Rb^+ as the only metal ion for 3 days, after which diffraction data were collected to 2.8 Å resolution. An F_o(DGD-Rb^+) − F_o(DGD-K^+) difference Fourier map is essentially featureless, apart from two strong spherical positive difference density peaks per subunit (Figure 2). The stronger of these peaks (13 standard deviations above the mean) is centered on the K^+ ion at site 1, indicating replacement of the K^+ ion by the heavier Rb^+ ion. A second prominent peak (eight standard deviations above the mean) is observed at metal binding site 2 on the surface of the DGD molecule, indicating (partial) binding of Rb^+ to this site as well. Metal binding site 2 is located in the proximity of a crystal packing contact, and the binding of Rb^+ to this site likely accounts for the slightly increased unit cell constants of DGD-Rb^+ compared to DGD-K^+.

The structure of DGD-Rb^+ was refined with Rb^+ ions at both metal binding sites. The Rb^+ ion at site 1 was refined with unit occupancy, resulting in a metal ion *B*-factor close to the average value of its ligand atoms (25 Å^2 compared to 22 Å^2). The refined site 1 structure in DGD-Rb^+ shows that binding of Rb^+ to this site does not lead to any significant changes compared to the DGD-K^+ structure. The Rb^+ ion

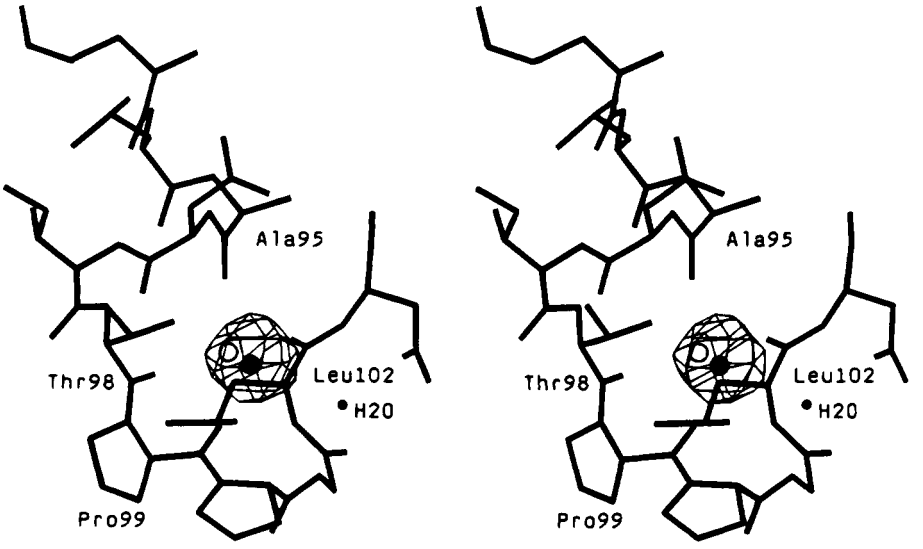

FIGURE 3: Stereoview of the F_o(DGD-Rb^+) − F_o(DGD-K^+) difference Fourier map in the region of metal binding site 2 in DGD-Rb^+. The map is contoured at a level corresponding to six standard deviations above the mean. The refined position of the Rb^+ ion is indicated by a large filled circle. For comparison, the position of the Na^+ ion in DGD-K^+ is also shown (open circle). A smaller filled circle represents a water molecule that is a metal ion ligand in DGD-Rb^+. All ligands to the Rb^+ ion are oxygen atoms, with an average metal−ligand distance of 2.9 Å. The distance between the Rb^+ ion and the nearest Asp103 carboxylate oxygen is 4.7 Å. The occupancy of the Rb^+ ion is only about 50% (see text).

is octahedrally coordinated by Asp307 $O_{\delta 1}$, the carbonyl oxygens of residues Leu78, Thr303, and Val305, the β-hydroxyl group of Ser80, and a water molecule at the upper axial position (see below). The average of the refined Rb^+−ligand distances is 2.74 ± 0.19 Å. Typical Rb^+−oxygen distances in small molecule crystal structures range from 2.7 to 3.1 Å, and an average value of 2.74 Å appears reasonable given the accuracy of a protein crystal structure at medium resolution. A significant fraction of remaining K^+ at site 1 in DGD-Rb^+ is unlikely in view of the low *B*-factor of a Rb^+ ion refined with full occupancy.

The structure of site 2 in DGD-Rb^+ is shown in Figure 3. The strong spherical peak in the F_o(DGD-Rb^+) − F_o(DGD-K^+) map around metal binding site 2 unambiguously shows that Rb^+ has replaced the Na^+ ion at site 2 to some extent in the DGD-Rb^+ crystal. The occupancy of a Rb^+ ion, placed at the maximum of the difference electron density peak and refined with a fixed *B*-factor of 25 Å^2, is ≈50 %. Since the Na^+ ion at site 2 appears to be difficult to remove (see above), it is very likely that the remaining 50% of the DGD-Rb^+ structure still contain a Na^+ ion at site 2, which will make a nonnegligible contribution to the averaged electron density at site 2 and thus preclude an accurate description of the Rb^+ coordination geometry.

Neglecting this complication, the coordination geometry of the Rb^+ ion can be described as a severely distorted octahedron, although it is not clear whether the Thr98 hydroxyl oxygen coordinates to the ion at all (d = 3.3 Å). The average metal−ligand distance is 2.9 Å (including the Thr98 $O_{\gamma 1}$−Rb^+ distance). The final difference Fourier map around site 2 in DGD-Rb^+ shows a weak positive peak on the solvent exposed side of the Rb^+ ion at a distance of 2.8 Å. This could be a weakly occupied, second water molecule bound to the metal center. Note that the effect of the electron density due to the remaining 50% Na^+ at site 2 in DGD-Rb^+ will be to "pull" the refined position of the Rb^+ ion closer to the position of the Na^+ ion. The observed distortion of the Rb^+ coordination octahedron is therefore not an artefact introduced by a partial Rb^+ occupancy. The refined protein structure of DGD-Rb^+ at metal binding site 2 is within experimental error identical to the DGD-K^+ structure, which has a Na^+ ion with full occupancy bound at this site. This shows that an exchange of Na^+ for Rb^+ at site 2 is notaccompanied by a significant change in the surrounding protein structure.

Comparison of the Structures of DGD with Li^+, Na^+, K^+, and Rb^+ Bound at Site 1. Figure 4 shows a superposition of the local structures around metal binding site 1 with Li^+, Na^+, K^+, and Rb^+ bound, respectively. The structures were superimposed using 39 Cα-atoms of the central seven-stranded β-sheet of the subunit that contributes the metal ion ligands at site 1 (rms deviations range from 0.08 to 0.18 Å). In this way, changes within the metal binding site structure are readily distinguishable from a possible change in quaternary structure upon ion exchange (as observed when K^+ is replaced by Na^+). From Figure 4, it is apparent that the structures of DGD with different alkali metal ions bound to site 1 near the active site fall into two distinct classes. Those with a large ion bound at site 1 (DGD-K^+ and DGD-Rb^+) are nearly identical. Likewise, the structures with small ions bound (DGD-Li^+ and DGD-Na^+) superimpose very well, despite the large difference in ionic radius (0.60 Å for Li^+ and 0.95 Å for Na^+) and dissimilar coordination geometries. However, the two discrete classes of structures display important differences that are described below.

A short qualifying remark may be needed here. Since the position of the Li^+ ion in DGD-Li^+ had to be modeled in the present work, any description of the *Li^+ coordination* can necessarily be only as valid as the model it is based on. However, many of the following arguments are based on experimentally determined *protein structures*, and the only assumption made there is that the DGD-Li^+ structure actually does have a Li^+ ion bound at site 1, an assumption that is in our opinion well justified (see above).

The first difference between the DGD-Li^+ and DGD-Na^+ structures on the one hand, as compared with the DGD-K^+ and DGD-Rb^+ structures on the other hand is the different number of water molecules that are coordinating to the alkali

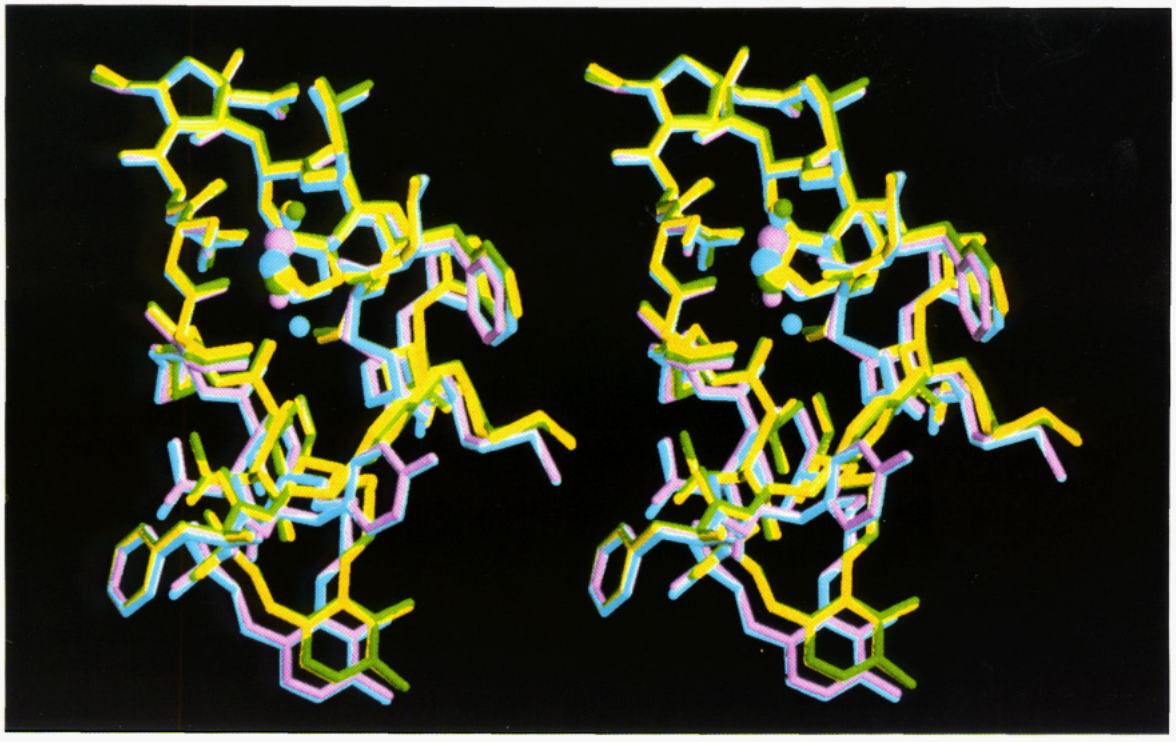

FIGURE 4: Superposition of four metal binding site 1 structures in an orientation similar to that in Figure 1 (from which the residue numbering can be obtained): DGD-Li^+ (purple), DGD-Na^+ (blue), DGD-K^+ (yellow), and DGD-Rb^+ (green). Metal ions and water molecules are represented by large and small spheres, respectively. The superposition was carried out using the 39 Cα atoms of the central β-sheet of the subunit that contains the metal binding site. Note the different side-chain conformations of Ser80 and Tyr301. The change in DGD quaternary structure (see text) is evident from the shifts of residues Gln52* and Lys272*-PLP of the other subunit (lower part of the figure). The figure was prepared with RIBBONS (Carson, 1987).

metal ion at site 1. All four structures contain an upper water molecule (in the view of Figures 1 and 4) at essentially the same position. In the DGD-Li^+ and DGD-Na^+ structures, however, a second water molecule replaces two metal ion ligands originating from the protein (the Ser80 side-chain hydroxyl group and the Val305 main-chain carbonyl oxygen), thereby reducing the coordination number of the alkali metal ion from six to four and five, respectively. This results in an altered local protein structure around metal binding site 1 that, in turn, leads to changes in both the DGD active site structure and the quaternary structure of the DGD tetramer.

Two residues in the active site region, Ser80 and Tyr301, have different side chain conformations in the two distinct DGD structures. The former is a ligand to the metal ion at site 1 in DGD-K^+ and DGD-Rb^+, and its replacement as a ligand by a water molecule in DGD-Li^+ and DGD-Na^+ causes the concerted reorientation of Ser80 and Tyr301. In DGD-K^+ and DGD-Rb^+, the aromatic side chain of Tyr301 is firmly held in place by a hydrogen bond to the active site residue Gln52*, whereas in DGD-Li^+ and DGD-Na^+, it is pointing away from Gln52* and has gained considerable mobility.

Apart from Ser80 and Tyr301, all other active site residues maintain their side-chain conformation upon an exchange of cations at site 1, although a few residues undergo moderate changes in their backbone conformation to accommodate the second water molecule at site 1 in the DGD-Li^+ and DGD-Na^+ structures. Metal binding site 1 in DGD is composed of two protein segments, about 220 residues apart in the sequence, that run antiparallel to each other. Interestingly, only one of these segments (comprising residues Phe300 to Val305) undergoes a significant structural change when a large ion at site 1 is exchanged for a smaller one; the other (residues Leu78 to Ser80) adjusts to only a minor extent.

One of the residues that undergoes a large (almost 1 Å) shift in its Cα atom position, is Thr303 (Figure 4). This residue provides two of the four hydrogen bonds that anchor the cofactor PLP of the adjacent subunit in the dimer to the protein, the other interactions being water-mediated. PLP is covalently linked to Lys272 and the shift of one of its main ligands contributed by the other subunit in the dimer presumably triggers the small (1°) rotation of the monomers with respect to each other. This rotation produces a certain amount of nonisomorphism between the two classes of DGD structures, which complicated the original structure determination of DGD (Toney *et al.*, 1993; Toney *et al.*, 1994), and which is also reflected in the scaling *R*-factors in Table 1. The very modest change in quaternary structure, however, is in itself unable to account for the antagonistic effects of small and large alkali metal ions on DGD activity. These have to be attributed to the specific rearrangements in the active site of the enzyme.

DISCUSSION

Ion Exchange at Metal Binding Site 2. The primary goal of this work was to understand how the binding of different alkali metal ions to site 1 near the active site affects the structure and enzymatic activity of DGD. However, this enzyme contains a second, structural, alkali metal ion binding site on the surface of the protein which also might be able to bind different alkali metal ions. Compared to site 1 which can accommodate all alkali metal ions, site 2 appears to be less promiscuous. In three structures (DGD-Li^+, DGD-Na^+, and DGD-K^+), a Na^+ ion is bound at site 2, and only Rb^+

could be shown to replace this Na^+ ion, albeit only partially. The preference of site 2 for Na^+ is hardly surprising. This metal binding site is made up of a tight and apparently very rigid reverse turn, which can be expected to display a rather strict preference for ions of just one size, analogous to the cation specificity of cyclic ionophores and cryptands (Dobler, 1982; Eisenman & Dani, 1987). The structure of site 2 with Rb^+ bound shows that the large Rb^+ ion (ionic radius 1.48 Å), in contrast to the smaller Na^+ ion (ionic radius 0.95 Å), does not fully enter the hydrophilic cavity of site 2, which cannot significantly adjust its structure to the larger ion. The Rb^+ ion therefore binds some 0.8 Å away from the position of the Na^+ ion, which leads to a distorted, rather unfavorable coordination geometry, and hence to a low affinity of site 2 for Rb^+. If one assumes that in the soaking experiment the Na^+/Rb^+ exchange had fully reached its equilibrium after three days, the occupancy of the Rb^+ ion allows the rough estimation of a dissociation constant of only 100 mM.

Cation Requirement of DGD. DGD requires a monovalent cation with an ionic radius of 1.3 to 1.5 Å (K^+, Rb^+, or NH_4^+) for full catalytic activity, in agreement with earlier observations (Aaslestad *et al.*, 1968). Only weak activation is observed with the smaller Na^+ and the larger Cs^+ ion. The lithium ion, as well as the alkaline earth metal ions are totally ineffective and cannot substitute for activating cations, although they appear to bind to the enzyme at the same location close to the active site (metal binding site 1). Binding to site 1 has so far been crystallographically demonstrated only for alkali metal ions,[4] but the pronounced inhibitory effect of divalent cations strongly suggests that they bind to the same site. The requirement of certain cations for DGD activity is not strict, since, at very high PLP concentrations, DGD is catalytically active in the absence of added activating cations.

The cation requirement of DGD closely parallels those of two other PLP-dependent enzymes, tryptophanase (Högberg-Raibaud *et al.*, 1975; Suelter & Snell, 1977) and tyrosine phenol-lyase (TPL; Toraya *et al.*, 1976), although DGD does not show a significant degree of sequence homology to either of these proteins. In tryptophanase and TPL, the essential monovalent cation has been shown to be important for the tight binding of the cofactor PLP (Toraya *et al.*, 1976). A similar role in DGD appears very likely, given the observation that high concentrations of PLP alone suffice for restoration of DGD activity after removal of activating cations. A prominent role of monovalent cations in the tight binding of the cofactor to DGD is also in accord with the crystal structure of DGD, where both cation and PLP are bound within the dimer interface in close proximity to each other (Toney *et al.*, 1993). Interestingly, the recently determined crystal structure of TPL (apoenzyme) did not reveal any protein-bound metal ions (Antson *et al.*, 1993). Based on the structures of DGD and TPL and their similar metal ion cofactor requirements, however, one can predict a metal ion binding site analogous to site 1 in DGD in the TPL holoenzyme structure. Indeed, in a preliminary report on the structure of holo-TPL, Antson *et al.* (1994) describe a K^+ binding site in the dimer interface in close proximity to the active site.

Inhibition by Nonactivating or Weakly Activating Ions. The inability of Li^+ and the alkaline earth metal ions (and to a lesser extent Na^+ and Cs^+) to activate metal-free DGD is a puzzling observation. In their presence DGD is catalytically inactive even when 1 mM PLP is added to the assay, that is under conditions where the metal-free enzyme is catalytically active (E. Hohenester and J. N. Jansonius, unpublished results). Thus, the inhibitory effect of Li^+ (and presumably also of Mg^{2+}, Ca^{2+} and Ba^{2+}) does not appear to be a result of a low affinity of DGD for PLP in the presence of these ions but must reside in the active site structural changes their binding to site 1 brings about, as already suggested in the first report on the DGD-K^+ and DGD-Na^+ structures (Toney *et al.*, 1993). Figure 4 shows that the binding of the activating cations K^+ or Rb^+ to site 1 yields almost identical active site structures. The same is true for the binding of the nonactivating cations Li^+ and Na^+, but the active site structures in their presence differ from those of DGD-K^+ and DGD-Rb^+ in several important respects (see Results). This striking correlation of the activating or inhibitory effect of a particular cation bound to site 1 with one of two possible active site structures adds much weight to our previous proposal that the rearrangement of the Ser80 and Tyr301 side chains is directly responsible for the inhibition of DGD by Li^+ and Na^+ (Toney *et al.*, 1993).

The structural changes on metal ion binding described here may affect the two DGD half-reactions, decarboxylation and transamination, differently. Detailed kinetic experiments to answer this question are underway. From models of catalytic intermediates, Tyr301 is proposed to be important in the binding of dialkylglycine substrates and catalytic intermediates (Toney *et al.*, 1993; Toney *et al.*, 1994), and it is tempting to speculate that its reorientation upon binding of a small, inhibitory alkali metal ion to metal binding site 1 affects the decarboxylation half-reaction more than the faster transamination reaction with its smaller substrate pyruvate.

Structural Switch at Site 1. The perhaps most intriguing observation in the study of alkali metal ion binding to DGD is the dramatic difference in the site 1 structures with either small (cation radius < 1.0 Å) or large (cation radius > 1.3 Å) alkali metal ions bound. In the detailed comparison of the DGD-Na^+ and DGD-K^+ structures (Toney *et al.*, 1994), a possible explanation for this structural switch has been given, which is substantiated and further extended by the present, more comprehensive experimental data.

Average alkali metal ion−ligand distances in small molecule structures range from 1.9 Å for Li^+ to 2.9 Å for Rb^+, with a concomitant increase in the preferred coordination numbers from five to eight and even higher, respectively (Shannon, 1976; Brown, 1988; Glusker, 1991). In this context, it should be noted that the alkali metal ion coordination numbers at site 1 in DGD are all at the low end of the range covered for the same ion in small molecule structures. This likely results from the fact that in a protein molecule the covalent linkage of the metal ion ligands imposes severe constraints on the coordination number and geometry. In order to accommodate alkali metal ions of all sizes at metal binding site 1, the protein structure clearly has to be rather flexible. At first glance, a simple *contraction*

[4] The crystal structure of DGD in the presence of 130 mM Cs^+ determined at 2.8 Å resolution (E. Hohenester and J. N. Jansonius, unpublished results) shows a Cs^+ ion with only partial occupancy at site 1. Furthermore, the side chains of Ser80 and Tyr301 appear to be disordered, and the structure of DGD-Cs^+ was therefore not included into the present discussion.

of the protein around the smaller ions Li^+ and Na^+, which would reduce the metal ion−ligand distances while essentially maintaining the coordination geometry of the metal ion, appears to be a possible solution to the problem. Closer inspection of the site 1 structure, however, shows that this would result in serious steric clashes between protein atoms. Instead, it is observed that two metal ligands from the protein are replaced by a single water molecule in the structures with Li^+ and Na^+ at site 1, thereby slightly *expanding* the protein structure around the metal ion. This allows for both optimal metal−ligand distances and coordination geometries in DGD-Li^+ and DGD-Na^+. The observation of two water ligands at site 1 in DGD-Li^+ and DGD-Na^+ compared to only one in DGD-K^+ and DGD-Rb^+ may also reflect the higher energy that is required to remove water molecules from the first hydration layer of small compared to large alkali metal cations (Hanzlik, 1976).

In conclusion, the two-state character of the metal ion size-dependent switch in the DGD active site structure appears to be a direct consequence of the insufficient flexibility of the metal ion ligand (*i.e.*, the protein molecule), which necessitates the insertion of a second water molecule at site 1 in DGD-Li^+ and DGD-Na^+. The insertion of this water molecule actuates the switch between two states of distinctly different local and global DGD structure in an all-or-none manner. While the *in vivo* significance of the metal ion switch in DGD remains unclear, the ion size dependency of the switch illustrates the crucial role alkali metal ions can play in organizing the active site structure and modulating the activity of an enzyme.

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