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# Effect of Potassium Ion on the Phosphorus-31 Nuclear Magnetic Resonance Spectrum of the Pyridoxal 5'-Phosphate Cofactor of *Escherichia coli* D-Serine Dehydratase[1]

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$^{31}$P NMR studies were undertaken to determine how potassium ion increases the cofactor affinity of *Escherichia coli* D-serine dehydratase, a model pyridoxal 5'-phosphate requiring enzyme that converts the growth inhibitor D-serine to pyruvate and ammonia. Potassium ion was shown to promote the appearance of a second upfield shifted cofactor $^{31}$P resonance at 4.0 ppm (pH 7.8, 25°C), that increased in area at the expense of the resonance at 4.4 ppm observed in the absence of $K^+$. $Na^+$ antagonized the $K^+$ promoted appearance of the second resonance. These observations suggest that $K^+$ and $Na^+$ stabilize conformational states that differ with respect to O-P-O bond angle, conformation, and/or hydrogen bonding of the phosphate group. An analysis of the dependence of the relative intensities of the two resonances on the $K^+$ concentration yielded a value of ca. 10 mM for the equilibrium constant for dissociation of $K^+$ from D-serine dehydratase. The chemical shift difference between the two resonances indicated that the $K^+$-stabilized and $Na^+$-stabilized forms of the enzyme interconvert at a frequency less than 16 $s^{-1}$ at pH 7.8, 25°C. © 1989 Academic Press, Inc.

Phosphorus-31 nuclear magnetic resonance ($^{31}$P NMR)[3] spectroscopy has been used to characterize cofactor interactions of pyridoxal 5'-phosphate (PLP) requiring enzymes including *Escherichia coli* D-serine dehydratase (DSD) a model $\alpha/\beta$ eliminase that converts the *E. coli* growth inhibitor D-serine to pyruvate and ammonia (for a review see Schnackerz (1)). The pH dependence of the cofactor phosphorus resonance is consistent with a p$K_a$ of about 6.4 for ionization of the 5'-phosphate group of the enzyme-bound cofactor which is bonded via a Schiff base linkage to the amino group of Lys-118 (2). The similarity of this p$K$ to that found in model low-molecular-weight PLP-amine Schiff bases has led Schnackerz *et al.* (2) to propose that the phosphate group of the enzyme-bound cofactor is accessible to solvent protons.

It should be noted, however, that PLP appears to be rigidly bound to the protein. Carbon-13 ($^{13}$C) NMR longitudinal relaxation time ($T_1$) measurements of the $\alpha$-carbon envelope indicate an overall rotational correlation time ($\tau_c$) for DSD of 25 ± 5 ns (at 33°C), a value close to that expected for a 48,000 $M_r$ protein (3). $^{13}$C NMR $T_1$ mea-

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[3] Abbreviations used: NMR, nuclear magnetic resonance; DSD, D-serine dehydratase; PLP, pyridoxal 5'-phosphate; $T_1$, longitudinal relaxation time; $T_2$, transverse relaxation time; $\tau_c$, rotational correlation time; Mops, 3-[*N*-morpholino]propanesulfonic acid; EAC, $\epsilon$-amino-*n*-caproic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; *S/N*, signal-to-noise ratio; CSA, chemical shift anisotropy.



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surements of the cofactor C5′ resonance indicate a rotational correlation time of 22 ± 5 ns for this atom. This result indicates that the motion of the C5′ carbon atom parallels that of the protein, suggesting that internal motion of this atom is restricted. Analogous $^{13}C$ NMR measurements on the C4′ resonance suggests that internal motion of this atom is also restricted. $^{31}P$ NMR measurements of line widths and longitudinal ($T_1$) and transverse ($T_2$) relaxation times of the cofactor resonance are also consistent with the view that PLP is rigidly held at the active site of DSD, and further suggest that large angle motion about the C5′–O bond of the enzyme-bound cofactor is restricted (2, 3).

Studies of the resolution of DSD by amines indicate that $K^+$ increases the affinity of DSD for its cofactor (4). In the present study we report that $K^+$ alters the chemical shift of the PLP phosphorus resonance in a manner consistent with a $K^+$-induced conformational change wherein interactions are altered which affect the O–P–O bond angle, conformation, and/or hydrogen bonding of the phosphate group of DSD-bound cofactor.

## EXPERIMENTAL

*Chemicals.* $^2H_2O$ was from ICN Biochemicals, Inc.; KCl was "Suprapur" grade from E. Merck. Fisher Scientific was the source of 85% $H_3PO_4$, HPLC grade. The following were from Sigma: NaCl ("Sigma" grade), PLP, 3-[*N*-morpholino]propanesulfonic acid (Mops), and ε-amino-*n*-caproic acid (EAC). Chelex-100 (100–200 mesh) and dithiothreitol (DTT) were obtained from Bio-Rad and Calbiochem, respectively. $TlNO_3$ and ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) were Aldrich "Gold Label" grade.

*NMR samples.* Samples of DSD were prepared from a strain of *E. coli* containing an expression plasmid for DSD (5). The concentration of DSD used in the NMR samples was between 113 and 625 μM. In addition to DSD the NMR samples contained 50 mM Mops, 1 mM DTT, and 2 mM EDTA. NMR studies on the Schiff base complex of PLP and EAC used samples with 50 mM Mops, 1 mM DTT, 2 mM EDTA, 110 mM EAC, and 3.4 mM PLP. The deuterium lock signal was given by 1% $^2H_2O$ added to each sample. Potassium and sodium as chloride salts were added as aliquots of 4 M stock solutions. The sample pH was checked before and after obtaining each NMR spectrum, and adjusted as necessary with 1 M HCl or 1 M NaOH. Final sample volumes were either 2.0 or 2.5 ml. Samples were placed in 10-mm flat bottom NMR tubes, and a Teflon vortex plug was positioned on top of the sample to prevent vortexing. Ultrafiltration of samples was performed using Centricon-10 microconcentrators from Amicon.

*NMR experiments.* NMR experiments were performed on a General Electric GN500 NB spectrometer. A 10-mm broad-band probe was used, tuned to phosphorus ($^{31}P$) at 202 MHz. Chemical shifts of the $^{31}P$ NMR spectra were referenced to external 85% $H_3PO_4$. Pulse widths were 60° or less, and the recycle time was ca. 1.0 s. Spectra were recorded with 8K data points over a spectral width of 8000 Hz, at 25°C. The number of acquisition scans ranged from ca. 30K to 80K, depending upon the concentration and sensitivity of the samples.

*Circular dichroism spectra.* Circular dichroism (CD) spectra were recorded on a Jasco Model 40C spectropolarimeter at 17°C using a cuvette of path length 1 cm. The sample consisted of 75 μM DSD and 20 mM $KH_2PO_4$ at pH 7.8.

*Data analysis.* NMR data were processed with added exponential line broadening of between 13 and 30 Hz. Line width analyses were performed assuming a Lorentzian line shape. The curve analysis and deconvolution program (GENCAP) also assumed a Lorentzian line shape. GENCAP was run under General Electrics GEN program on a Nicolet 1280 computer.

## RESULTS

Figure 1 illustrates the effect of $K^+$ on the $^{31}P$ resonance of DSD-bound cofactor at pH 7.8, 25°C. Addition of $K^+$ resulted in the appearance of a second upfield shifted resonance at 4.0 ppm that increased in area at the expense of the resonance at 4.4 ppm observed in the absence of $K^+$. A similar effect of $K^+$ was observed at pH 7.0 where the two resonances appeared at 4.0 ppm (no $K^+$) and 3.4 ppm. Removal of $K^+$ from a DSD sample by ultrafiltration resulted in disappearance of the $K^+$-dependent resonance and reappearance of the original resonance ($\Delta\nu_{1/2}$ = 27 Hz). Upon readdition of $K^+$ to the sample, the $K^+$-dependent resonance reappeared. These observations indicate that the $K^+$-induced effects on the $^{31}P$ resonances were reversible.

No change in the $^{31}P$ chemical shift was produced by addition of 40 mM NaCl (Fig. 2). Thus, it is unlikely that the observed

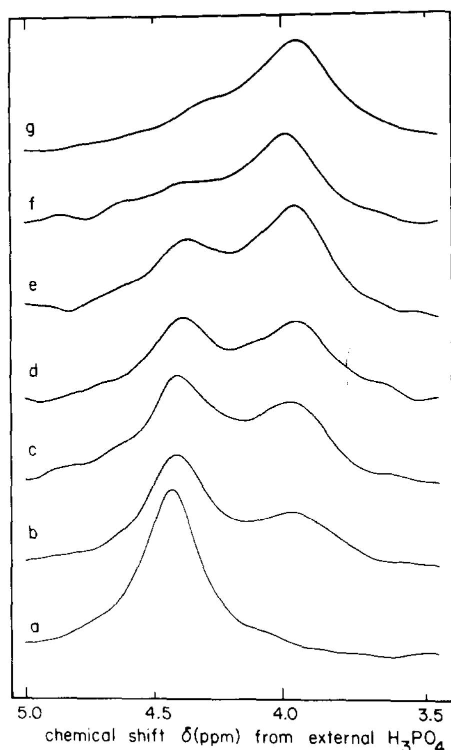



FIG. 1. Effect of $K^+$ on the $^{31}P$ NMR signal of pyridoxal 5′-phosphate in D-serine dehydratase (340 μM). (a) No $K^+$; (b) 2.5 mM KCl; (c) 5.0 mM KCl; (d) 7.5 mM KCl; (e) 12.5 mM KCl; (f) 20 mM KCl; (g) 40 mM KCl. Samples were in 50 mM Mops, 1 mM DTT, 2 mM EDTA, pH 7.8, at 25°C. The spectra were processed with an exponential line broadening of 25 Hz.

effects of $K^+$ were due to alterations in ionic strength. Although $Na^+$ did not detectably affect the $^{31}P$ chemical shift it broadened the resonance, and noticeable line broadening was observed in the presence of only 7.5 mM NaCl. Addition of 300 mM KCl produced an increase in line width ($\Delta\nu_{1/2}$ = 42 Hz) of the $K^+$-dependent resonance (at 4.0 ppm) comparable to that produced by 40 mM NaCl on the signal at 4.4 ppm. In addition to broadening the $^{31}P$ resonance seen in the absence of $K^+$, $Na^+$ also reduced the effectiveness of $K^+$ in producing the second resonance. For example, in the presence of 40 mM $Na^+$ a concentration of more than 13 mM $K^+$ was required to raise the intensity of the $K^+$-dependent signal to that seen in the presence of 7 mM $K^+$ in the absence of $Na^+$. Moreover, addition of 160 mM NaCl to DSD (at pH 7.8) in the presence of 50 mM KCl (where the upfield $K^+$-dependent resonance is the predominant signal) resulted in appearance of the original resonance at 4.3 ppm (Fig. 2). Taken together these effects suggest that both $Na^+$ and $K^+$ bind to DSD but stabilize different forms of the enzyme-bound cofactor.

Essentially indistinguishable CD spectra of DSD in the region of the cofactor chromophore (410 nm) were obtained in pH 7.8, 50 mM Mops buffer in the absence of added salt, with 0.3 M KCl, and in the presence of 0.3 M NaCl. This observation

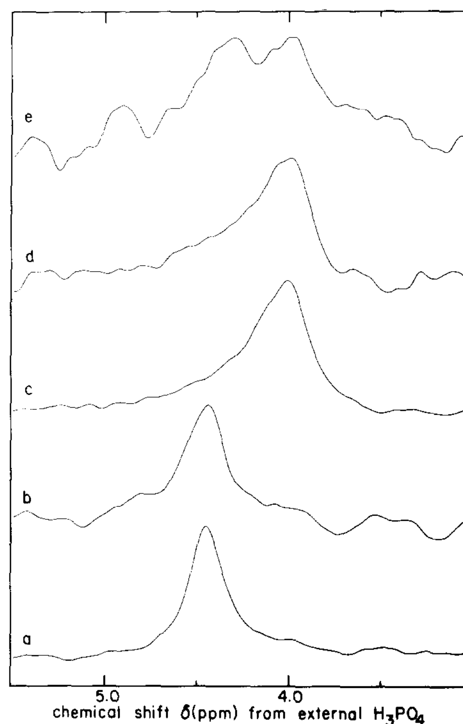



FIG. 2. Comparison of the competing effects of $Na^+$ and $K^+$ on the $^{31}P$ NMR signal of pyridoxal 5′-phosphate in D-serine dehydratase. (a) No added $Na^+$ or $K^+$; (b) 40 mM NaCl; (c) 60 mM KCl; (d) 40 mM NaCl + 50 mM KCl; (e) 160 mM NaCl + 50 mM KCl. Experimental conditions were the same as for Fig. 1 except that 625 μM enzyme was used for spectrum c. The spectra presented here were processed with an exponential line broadening of 20 Hz.

suggests that these monovalent cations have little effect on the interactions between the cofactor pyridine ring and the protein. Thus, the different effects on the $^{31}P$ spectra seen with $K^+$ and $Na^+$ are probably limited to a difference in their influence upon the phosphate group of PLP.

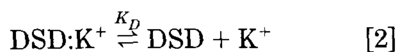
The presence of two peaks in the NMR spectrum whose areas respectively increase and decrease with increasing concentrations of $K^+$ suggests that the two peaks are in slow exchange on the NMR time scale. Under slow exchange conditions

$$\nu_e \leqslant |\nu_A - \nu_B|/5 \qquad [1]$$

where $\nu_e$, $\nu_A$, and $\nu_B$, respectively, represent the exchange frequency, and the frequencies for resonances A and B (see e.g., (6)). This equation yields upper limits of 16 and 24 $s^{-1}$ for the frequency of interconversion of the two states at pH 7.8 and pH 7.0, respectively.

For purposes of comparison we determined the effect of $K^+$ and $Na^+$ on the $^{31}P$ resonance for a PLP:$\epsilon$-amino caproic acid Schiff base, a model for the Schiff base linking PLP to Lys-118 in DSD. Addition of either 300 mM NaCl or KCl to PLP in pH 7.8, 50 mM Mops buffer resulted in a downfield shift in the $^{31}P$ resonance from 4.2 to 4.3 ppm.

To analyze the effect of $K^+$ on the $^{31}P$ spectra of DSD, the resonances were resolved and their areas determined using the GENCAP program (Table I). The dependence of the area under the $^{31}P$ resonances on the concentration of $K^+$ was analyzed by assuming that binding of $K^+$ to DSD alters the chemical shift of the $^{31}P$ resonance of DSD bound PLP. If we assume the following equilibrium for binding of $K^+$,



$$\text{DSD:K}^+ \overset{K_D}{\rightleftharpoons} \text{DSD} + \text{K}^+ \qquad [2]$$

it follows that

$$[\text{K}^+]/f = [\text{K}^+] + K_D \qquad [3]$$

where $f$ represents the fraction of the $^{31}P$ signal attributable to DSD:$K^+$, and $K_D$ is the equilibrium constant for dissociation of $K^+$ from DSD. Thus, a plot of $[K^+]/f$ versus $[K^+]$ should yield a straight line with a slope of unity. The data in Table I and Fig. 3 illustrate the fit of the data to Eq. [3] and suggest a value of about 10 mM for $K_D$.

In an attempt to determine whether the monovalent cations might interact directly with the 5'-phosphate group of the cofactor, the effect of $Tl^+$ on the phosphate resonance was studied, since $^{203}Tl^+$ (natural abundance 29.5%) and $^{205}Tl^+$ (natural abundance 70.5%), both with a spin of $\frac{1}{2}$, when directly bonded to the phosphate might (depending upon the ionic character of the interaction) split the phosphorus resonance. Interestingly, addition of 100 mM $TlNO_3$ produced an upfield shift to 4.2 ppm of the $^{31}P$ signal of PLP bound to DSD. No coupling with $Tl^+$ could be detected, however. Since $Tl^+$ complexes with a high ionic character might not show a $Tl^+$ coupled $^{31}P$ resonance (10), we cannot rule out a direct interaction with phosphate. Consistent with this conclusion addition of $Tl^+$ to free PLP in Mops buffer at pH 7.8 did not split the phosphate resonance, but, indicative of an interaction with the phosphate group, $Tl^+$ shifted the phosphate resonance slightly downfield.

## DISCUSSION

$K^+$ in contrast to $Na^+$ has been reported to increase the cofactor affinity of several pyridoxal 5'-phosphate requiring enzymes including tryptophanase (11, 12), L-threonine dehydratase (13), and D-serine dehydratase (4). The thermodynamic linkage of the binding of $K^+$ and cofactor has been analyzed quantitatively in the case of DSD, in studies of the effects of $K^+$ on the rate and extent of resolution of DSD by 2-hydroxyethylamine (4). These studies indicate that binding of $K^+$ to DSD increases its affinity for PLP by at least 13-fold. The simplest explanation for the two $^{31}P$ resonances reported in the present paper is that DSD exists in at least two states that differ with respect to interactions involving the 5'-phosphate of PLP.

The dispersion in the values (5–15 mM) for the equilibrium constant for dissocia-

TABLE I

$^{31}P$ NMR DATA AND ANALYSIS FOR $K^+$ ASSOCIATION WITH D-SERINE DEHYDRATASE

| | [KCl]/$f_b$ | | | | | |
|---|---|---|---|---|---|---|
| | Set a | | Set b | | Set c | |
| [KCl] (mM) | Data | Fit | Data | Fit | Data | Fit |
| 2.5 | 7.4 | 8.8 | | | | |
| 5.0 | 13.8 | 12.4 | 14.0 | 19.1 | 11.8 | 13.5 |
| 7.5 | 14.9 | 16.1 | | | 12.1 | 15.9 |
| 10.0 | 19.2 | 19.7 | 23.6 | 23.5 | | |
| 12.5 | 18.7 | 23.4 | | | 21.4 | 20.7 |
| 15.0 | 32.5 | 27.0 | | | | |
| 20.0 | 36.5 | 34.3 | 31.1 | 32.4 | 27.1 | 27.9 |
| 40.0 | 62.1 | 63.5 | 59.5 | 50.1 | | |
| 50.0 | | | | | 67.4 | 56.8 |
| 100.0 | | | 100.0 | 103.3 | 100.0 | 104.8 |
| Slope | 1.5 (±0.1) | | 0.89 (±0.08) | | 0.96 (±0.08) | |
| Intercept (mM) | 5 (±2) | | 15 (±4) | | 8 (±4) | |
| $r^2$ | 0.971 | | 0.974 | | 0.976 | |

*Note.* Set a, 340 μM DSD, pH 7.8; Set b, 222 μM DSD, pH 7.8; Set c, 222 μM DSD, pH 7.0. The data were fit to the following equation: $[KCl]/f_b = m[KCl] + b$; where $f_b$ is the fraction of the $^{31}P$ NMR signal associated with the addition of KCl as determined from the areas under the two $^{31}P$ NMR resonances. The theoretical values of $m$ and $b$ are unity and the dissociation constant (mM) of $K^+$ from DSD, respectively. Linear least-squares fit results are given below each data set, and the errors are 1 SD ($\sigma_{n-1}$).

tion of $K^+$ from DSD reported in the present paper is attributed to the low *S/N* of the spectra. Considering the precision, the estimates of $K_D$ obtained from the NMR measurements are in tolerable agreement with the value of 23 mM obtained for $K_D$ from an analysis of the dependence on $K^+$ concentration of the rate constant for resolution of DSD by 2-hydroxyethylamine (4). The antagonistic effect of $Na^+$ on the $K^+$-induced $^{31}P$ resonance is consistent with the view that $K^+$ and $Na^+$ stabilize different states of the enzyme, with the $K^+$-stabilized state having the higher affinity for PLP.

The major relaxation mechanism of $^{31}P$ nuclei in macromolecules at higher fields has been shown (16, 17) to be chemical shift anisotropy (CSA). In the previous $^{31}P$ NMR work on DSD at lower field strengths (2, 3) dipole–dipole interactions were the predominant relaxation mechanism, and line widths were reported of 10–14 Hz (2) at 72.9 MHz and 11–12 Hz (3) at 40.5 MHz. Thus, the line widths observed in this study (27–42 Hz) at 202 MHz are undoubtedly larger due to the predominance of the CSA relaxation mechanism at this higher field.

The downfield shift observed for the $^{31}P$ resonance of the PLP:ε-amino caproic acid Schiff base in Mops buffer produced upon the addition of NaCl or KCl probably reflects altered hydrogen bonding interactions when the cation penetrates and disrupts the hydration shell of the phosphate group (14). Similar downfield chemical shifts have been reported when alkali metal monocations are added to orthophosphate and various mono- and di-anionic phosphate esters (14, 15).

The observation that addition of $K^+$ to PLP model compounds and other phosphate derivatives results in a downfield shift in the $^{31}P$ resonance, whereas $K^+$ induces an upfield shift of the phosphate $^{31}P$

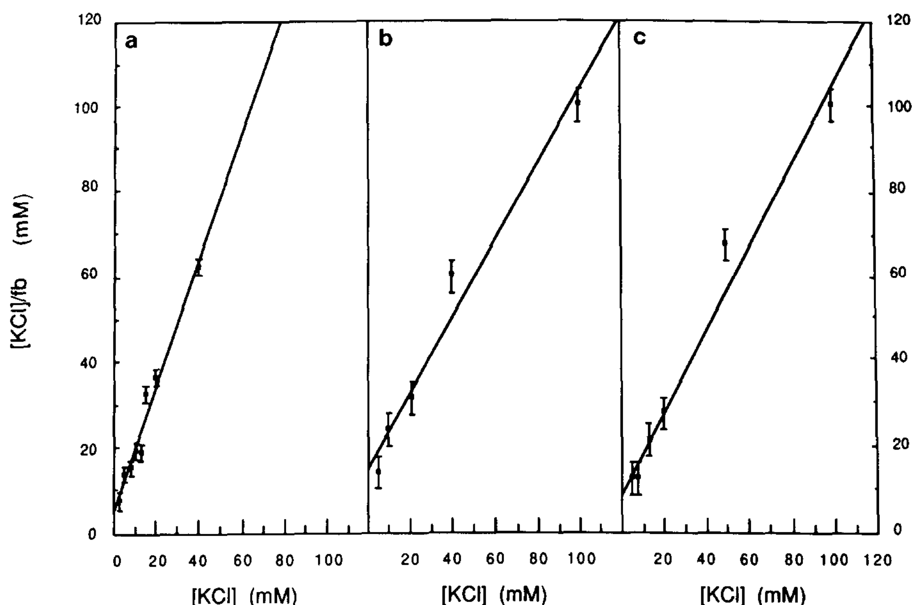



FIG. 3. Determination of the equilibrium constant for the binding of $K^+$ to D-serine dehydratase. (a) 340 μM DSD, pH 7.8; (b) 222 μM DSD, pH 7.8; (c) 222 μM DSD, pH 7.0. Other experimental parameters are as in Fig. 1. The data were fit to the following equation: $[KCl]/f_b = m[KCl] + b$; where $f_b$ is the fraction of the $^{31}P$ NMR signal associated with the addition of KCl as determined from the areas under the two $^{31}P$ NMR resonances. The theoretical values of $m$ and $b$ are unity and the dissociation constant of $K^+$ from DSD, respectively. The values of $m$ and $b$ as determined with a linear least-squares fit are given in Table I. The error bars represent 1 SD ($\sigma_{n-1}$) from the corresponding calculated data fit. Solid lines represent the linear least-squares fit of the data to the above equation.

signal of PLP-bound DSD indicates the existence of an interaction(s) between DSD and the phosphate group of the bound cofactor. Because the effects of the protein interaction on the $^{31}P$ resonance of PLP are unknown it is presently not possible to determine whether $K^+$ and $Na^+$ directly interact with the phosphate group of the enzyme-bound cofactor. It is tempting to speculate that occupancy of a monovalent cation binding site of DSD by $K^+$, but not by $Na^+$, affects the O-P-O bond angle(s) or conformation or hydrogen bonding of the cofactor phosphate group. The ability of $Na^+$ to alter the interactions of the phosphate group upon binding to the cation binding site may be due to the hydrated ionic radius of $Na^+$ (3.58 Å) being larger than that of $K^+$ (3.31 Å) (18). Consistent with this view is the fact that $Tl^+$, which causes an upfield shift of the $^{31}P$ signal of DSD like $K^+$, has a hydrated ionic radius (3.30 Å) similar to that of $K^+$. It is anticipated that X-ray diffraction analysis will reveal the structural basis for the ability of $K^+$ and $Na^+$ to stabilize different forms of DSD.

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