Phosphate Ion Partially Relieves the Cooperativity of Effector Binding in D-3-Phosphoglycerate Dehydrogenase without Altering the Cooperativity of Inhibition†

Gregory A. Grant,*,‡,§ Xiao Lan Xu,‡ Zhiqin Hu,‡ and Angie R. Purvis‡

Department of Molecular Biology and Pharmacology and Department of Medicine, Washington University School of Medicine, 660 South Euclid Avenue, Box 8103, St. Louis, Missouri 63110

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ABSTRACT: The binding of L-serine to phosphoglycerate dehydrogenase from *E. coli* displays elements of both positive and negative cooperativity. In addition, the inhibition of enzymatic activity by L-serine is also cooperative with Hill coefficients greater than 1. However, phosphate buffer significantly reduces the cooperative effects in serine binding without affecting the cooperativity of inhibition of activity. The maximal degree of inhibition and fluorescence quenching in Tris buffer occurs when an average of two serine binding sites out of four are occupied. This value increases to three out of the four sites at maximal levels of inhibition and quenching in phosphate buffer. The increase from two to three sites appears to be due to the ability of phosphate to reduce the site to site cooperative effects and render each ligand binding site less dependent on each other. The correlation between the level of inhibition and the fractional site occupancy indicates that in Tris buffer, one serine is bound to each interface at maximal effect. In the presence of phosphate, the order of binding appears to change so that both sites at one interface fill before the first site at the opposite interface is occupied. In each case, there is a good correlation between serine binding, conformational change at the regulatory site interfaces, and inhibition of enzyme activity. The observation that phosphate does not appear to have a similar effect on the cooperativity of inhibition of enzymatic activity suggests that there are two distinct cooperative pathways at work: one path between the four serine binding sites, and one path between the serine binding sites and the active sites.

D-3-Phosphoglycerate dehydrogenase (PGDH) (EC 1.1.1.95) from *E. coli* is an allosterically regulated enzyme of the V-type (*1*, *2*) that is composed of four identical subunits (*3*). Each subunit contains three structural domains: the substrate binding domain, the nucleotide binding domain, and the regulatory domain. Each regulatory domain contacts the regulatory domain of an adjacent subunit, producing two regulatory domain interfaces found at opposite ends of the tetramer. This interface forms the effector ligand binding sites for L-serine, an allosteric inhibitor of PGDH. Serine forms hydrogen bonds between the adjacent domains, and each of the two regulatory domain interfaces binds two L-serine molecules with 180° symmetry.

Previously, we have shown (*4*) that binding of two of the four serines is all that is necessary to produce maximal inhibition. Furthermore, the binding of the second serine displays significant positive cooperativity while negative cooperativity is evident in the binding of the last two serines.

This report demonstrates that phosphate ion has the effect of relieving, to a large extent, the cooperativity of serine binding and that this can be separated from the cooperativity of inhibition. Fluorescence quenching data demonstrate that there is a direct relationship between the site occupancy, the conformational change at the regulatory site interfaces, and the inhibition of activity. Although the molecular basis for this "phosphate effect" is unknown, the data confirm that serine binding at both interfaces is required for maximal inhibition.

MATERIALS AND METHODS

PGDH was expressed, isolated, and assayed as previously described (*5*, *6*). Activity was determined at constant temperature using α-ketoglutarate (*7*) as the substrate and by monitoring the decrease in absorbance of NADH at 340 nm (*8*). Protein concentration was determined initially by the Bradford method as previously described (*9*, *10*) and by quantitative amino acid analysis for the serine binding studies. All experiments are conducted with $PGDH_{4C/A}$, which is a form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine. This construct has been described previously (*10*) and is used here for consistency of comparison to past studies. Kinetically, native PGDH and $PGDH_{4C/A}$ are very similar (*10*). $PGDH_{4C/A}$ E360W is a mutant that has also been previously described (*10*), in which a tryptophan residue has been placed at the regulatory domain interface and whose fluorescence emission is quenched by serine binding.

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* Corresponding author: Department of Molecular Biology and Pharmacology, Box 8103, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110. Phone: 314-362-3367. FAX: 314-362-4698. Email ggrant@molecool.wustl.edu.

‡ Department of Molecular Biology and Pharmacology.

§ Department of Medicine.

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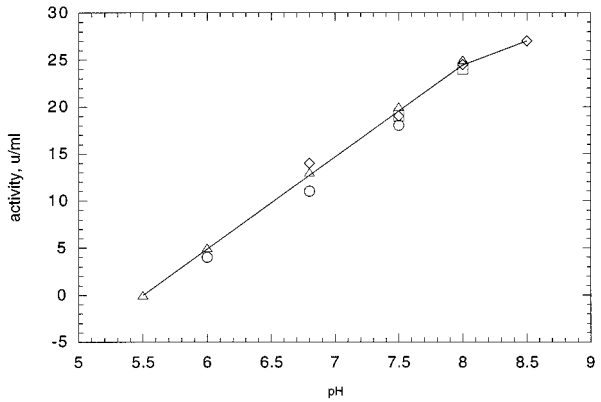

FIGURE 1: Activity of PGDH as a function of pH. The activity of PGDH was determined in Tris (◇), imidazole (○), borate (□), and phosphate (△), buffers at the indicated pH.

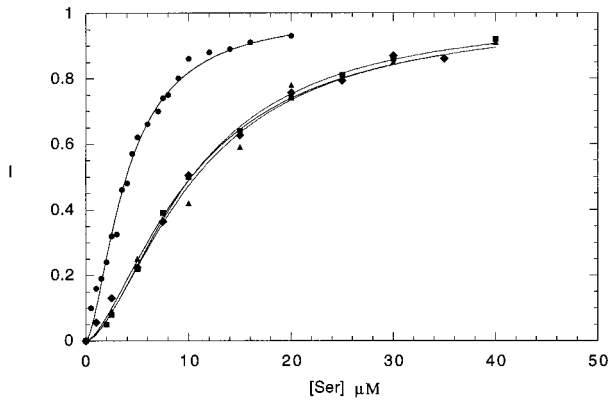

FIGURE 2: L-Serine inhibition of PGDH. The fractional inhibition of enzyme activity is plotted versus L-serine concentration (μM) for Tris (■), imidazole (▲), borate (◆), and phosphate (●) buffers. The solid lines are produced by fitting the data to the Hill equation.

Equilibrium dialysis was performed in 500 μL dialysis cartridges obtained from Sialomed, Inc. (Columbia, MD). Dialysis was performed in the designated buffer for 16 h with [^{3}H]-L-serine as a tracer in appropriate concentrations of unlabeled L-serine. Cells were sampled in triplicate, and the average of 10 min counts was used to calculate concentrations of free and bound L-serine. The nominal PGDH concentration was 5 μM tetramer in all binding experiments. Amino acid analysis was used to determine the actual concentration. Binding experiments were performed in triplicate with different enzyme preparations. When simultaneous binding was performed under different buffer conditions, enzyme was dialyzed against water for 24 h with two changes of dialysis solution, and the dialyzed protein was split into two equal aliquots. Each aliquot was diluted to 5 μM protein and 20 mM buffer with either potassium phosphate, pH 7.5, or Tris, pH 7.5, and equilibrium dialysis was performed simultaneously with identical L-serine preparations.

Experimental data were fit to equations with the curve-fitting program of Kaleidograph (Synergy Software). Coefficients of cooperativity and apparent dissociation constants for serine inhibition were determined by fitting the inhibition data to the Hill equation (*4*, *11*). Adair constants were determined by fitting the serine binding data to the Adair equation for a molecule with four binding sites (*4*, *11*) where Y is the fraction of sites occupied per total number of sites and K_i are the stepwise Adair constants expressed as dissociation constants. Intrinsic site dissociation constants were calculated from the Adair constants using the appropriate statistical relationships (*4*, *11*).

Fluorescence quenching of $PGDH_{4C/A}$ E360W by serine binding was analyzed by the method of Lohman and Bujalowski (*14*). Fluorescence intensities and ligand concentrations were corrected for volume changes.

RESULTS

The pH range over which PGDH is active is between 6.0 and 8.5 (Figure 1). Below pH 6.0 the activity is too low to be accurately measured. It increases in a nearly linear manner as pH is increased until just above pH 8.5 where the enzyme activity falls off dramatically. Identical pH profiles are found regardless of the buffer (Figure 1), indicating that there is no apparent effect on activity by the nature of the buffer salts.

Table 1: Properties of L-Serine Inhibition of PGDH in Various Buffers

buffer (20 mM, pH 7.5)	Hill coefficient (n)	Hill constant (K)
Tris	1.67 ± 0.04	49.4 ± 4.5
imidazole	1.62 ± 0.10	46.9 ± 12.2
borate	1.56 ± 0.06	38.0 ± 6.2
phosphate	1.62 ± 0.07	9.2 ± 1.0

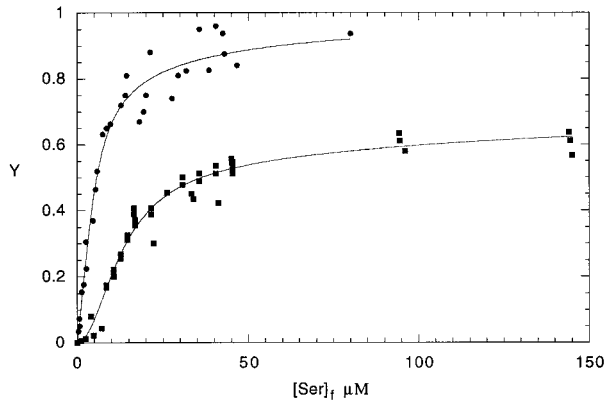

FIGURE 3: L-Serine binding to PGDH. The fractional occupancy of the tetramer (Y) is plotted versus the free L-serine concentration (μM). The solid lines are the data fit to the Adair equation for four binding sites. 20 mM phosphate, pH 7.5 (●); 20 mM Tris, pH 7.5 (■). The data for Tris buffer are taken from ref *4*.

At pH 7.5, the inhibition profiles of PGDH activity by L-serine in most buffers tested (Tris, borate, and imidazole buffer) are essentially the same (Figure 2). However, inhibition in phosphate buffer is more sensitive to serine concentration, with 90% inhibition occurring at approximately 10 μM L-serine as compared to approximately 40 μM for the other buffers. Fitting the inhibition data to the Hill equation (solid lines in Figure 2) yields coefficients of cooperativity greater than 1 (Table 1), indicating that similar positive cooperativity is observed in all cases.

When direct measurement of L-serine binding by equilibrium dialysis is compared, there is an increase in the stoichiometry of serine binding in phosphate buffer over that observed in Tris buffer (Figure 3). Not only does serine bind more readily at lower concentrations in phosphate buffer,

Table 2: Dissociation Constants (μM) for L-Serine Binding to PGDH[a]

	20 mM Tris, pH 7.5[b]		
K_1	$53.3 \pm (1.3 \times 10^{-4})$	K'_1	213
K_2	$2.8 \pm (2.6 \times 10^{-3})$	K'_2	4.2
K_3	$83.9 \pm (5.3 \times 10^{-5})$	K'_3	55.9
K_4	very large	K'_4	very large
	20 mM Phosphate, pH 7.5		
K_1	2.5 ± 1.8	K'_1	10
K_2	7.6 ± 12	K'_2	11.4
K_3	3.1 ± 4.1	K'_3	2.1
K_4	33.6 ± 14.1	K'_4	8.4

[a] Expressed as dissociation constants (μM). Adair constants are denoted as K_i, and intrinsic site dissociation constants are denoted as K'_i. [b] Values for Tris buffer are taken from reference *4*.

but more total serine binding is observed at higher serine concentrations as well. To determine if this was an effect of the Tris or the phosphate, binding was also performed in 20 mM borate buffer, pH 7.5, and 20 mM imidazole buffer, pH 7.5. The stoichiometry of L-serine binding in borate and imidazole buffers is similar to that seen for Tris (not shown). This apparent difference in binding behavior between Tris and phosphate buffer was verified by performing the binding simultaneously in both buffers with the same enzyme preparation. The result was as shown in Figure 3 where binding in phosphate is essentially stoichiometric while binding in Tris was maximal at less than stoichiometric proportions. This ruled out relative errors in protein concentration, enzyme specific activity, and homogeneity and confirmed that the difference in serine binding in phosphate buffer is real.

As reported previously for Tris buffer (*4*), fitting the binding data to the Adair equation yields dissociation constants that indicate significant positive cooperativity for binding of the second serine ligand and appreciable negative cooperativity for binding the third and particularly the fourth serine ligands (Table 2). When serine binding is done in phosphate buffer, the negative cooperative effect is absent and the positive cooperative effect appears to be greatly diminished. Although the error in the dissociation constants determined in phosphate buffer is too large to definitively conclude that there is a low level of positive cooperativity remaining, the Scatchard plot of the phosphate binding data (Figure 4) produces a slightly concave profile indicating some degree of positive cooperativity exists.

Figure 5 plots the inhibition of activity versus the site occupancy in both buffers. As previously demonstrated, in Tris buffer the enzyme is 90% inhibited when only an average of two sites are occupied and 50% inhibited when only one site is occupied. On the other hand, in phosphate buffer the 90% level of inhibition is now reached when an average of three sites are occupied. This determination is based on direct experimental observation of the binding and inhibition and does not rely on curve fitting or the determination of binding constants derived from curve fitting.

Serine binding was also measured by fluorescence quenching using PGDH E360W (*10*). The data were analyzed by the method of Lohman and Bujalowski (*12*), which is a model-independent thermodynamic method for determining binding isotherms. Since the quenching of intrinsic protein fluorescence is monitored, the method is independent of the direct measurement of site occupancy. In this case, the

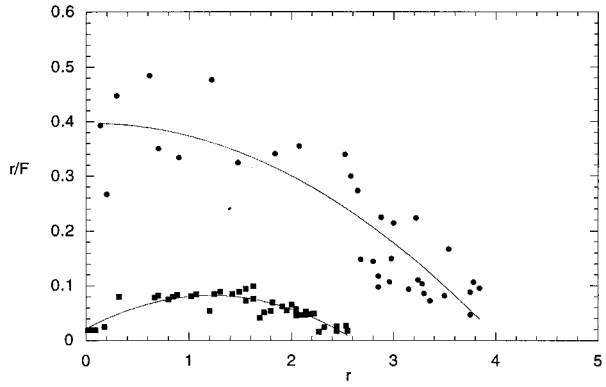

FIGURE 4: Scatchard plot of L-serine binding data. *r* is the number of serines bound per tetramer, and *r*/*F* is *r* divided by the free serine concentration (μM). 20 mM phosphate, pH 7.5 (●); 20 mM Tris, pH 7.5 (■). The data for Tris buffer are taken from ref 4.

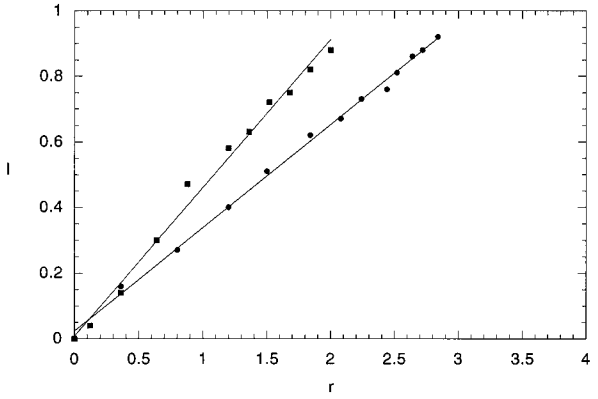

FIGURE 5: Relationship of bound serine to degree of inhibition of PGDH. Number of serines bound per tetramer (*r*) is plotted against the fractional inhibition of the enzyme for phosphate buffer (●) and Tris buffer (■). The data for Tris buffer are taken from ref *4*.

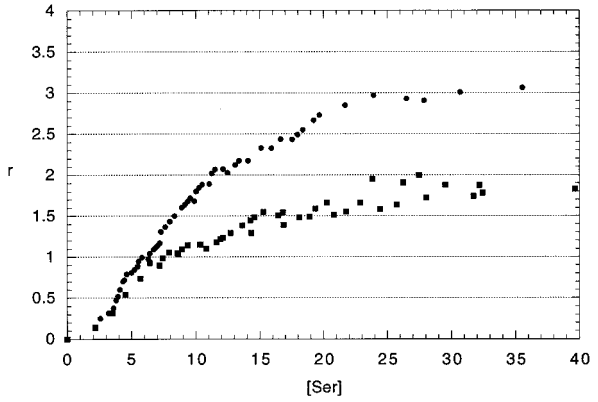

FIGURE 6: Relationship of bound serine to degree of fluorescence quenching. Number of serine bound per tetramer (*r*) is plotted against the free serine concentration (μM) for phosphate buffer (●) and Tris buffer (■).

fluorescence quenching is measuring a conformational change of the regulatory domains in response to serine binding. The method allows the number of serines bound at full quenching to be determined. The results of this analysis are presented in Figure 6 and clearly show that two serines are bound at full quenching in Tris buffer but that three serines are bound at full quenching in phosphate buffer. These data are consistent with the results of the direct serine binding data shown in Figure 5. The percent of total

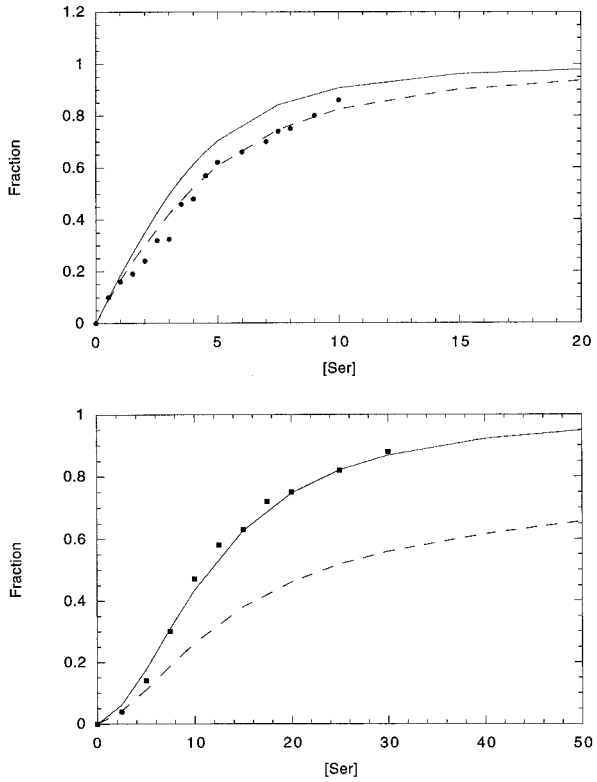

FIGURE 7: Comparison of experimental and predicted inhibition patterns. Experimental serine inhibition data are plotted as the symbols (●) and (■). The solid line represents the hypothetical inhibition curves predicted from the distribution of bound species for $I \approx (0.5P_1 + P_2 + P_3 + P_4)$. The dashed line represents that for $I \approx (0.5P_1 + 0.5P_2 + P_3 + P_4)$. Top: Phosphate buffer. Bottom: Tris buffer. The data for Tris buffer are taken from ref *4*.

fluorescence that is quenched is 53% in Tris buffer and 48% in phosphate buffer, indicating that approximately the same number of tryptophan side chains are being quenched in each case. This is also consistent with the conclusion that all four tryptophan side chains at the regulatory domain interfaces (two at each interface) are being quenched. There are a total of four tryptophans in the native tetramer that are not quenched by serine. Addition of the four tryptophans at position 360 increased the fluorescence yield of the protein by a factor of 2, and serine-dependent quenching produces approximately a 50% decrease in fluorescence signal.

It was previously shown that, in Tris buffer, each interface affected approximately two active sites (*4*). That is, the inhibition pattern could be predicted by the site occupancy and the distribution of species with one, two, three, or four bound serines. If each of the first two serines binds at opposite interfaces, the inhibition of activity which results would be expected to be predicted by $I \approx (0.5P_1 + P_2 + P_3 + P_4)$, where P_i is the fraction of each species. If the first two serines bind at the same interface, the inhibition pattern would be predicted by $I \approx (0.5P_1 + 0.5P_2 + P_3 + P_4)$. Binding in Tris buffer followed the former case and led to the conclusion that the first two serines bind at opposite interfaces. As shown in Figure 7, binding in phosphate buffer follows the latter case and suggests that both of the first two serines bind at the same interface and binding of the third serine at the other interface is necessary for optimal inhibition. This is consistent with the tethered domain model and the data presented in Figure 5.

DISCUSSION

The data indicate that L-serine binding by PGDH appears to be uniquely sensitive to phosphate ion and that its presence changes the enzyme's allosteric characteristics. The degree of inhibition at a particular serine concentration is increased, serine site occupancy increases, and the cooperative nature of serine binding decreases. At the same time, however, the level of cooperativity seen in the inhibition of activity remains essentially unchanged. The data also demonstrate that serine site occupancy correlates equally to the level of inhibition of activity and fluorescent quenching, two phenomena which occur at distinctly different parts of the enzyme. However, two sites in Tris buffer and three sites in phosphate buffer are occupied at maximal levels of inhibition and fluorescence quenching.

The crystal structure with serine bound to the enzyme shows that the serine molecules are buried in the regulatory domain interface and not accessible to solvent. Presumably, the regulatory domains separate to some extent to allow serine access to its binding sites from the solvent. Unfortunately, a crystal structure of the active enzyme in the absence of serine is not available. This partial opening and closing of the regulatory domain interface is thought to be responsible for the fluorescence quenching of Trp 360 since it has been demonstrated that the quenching is mediated by an amino acid side chain on the adjacent subunit (*10*).

A simplified view of the mechanism would be that serine enters an "open" regulatory site interface which "closes" when serine binds, and that in turn produces quenching of the fluorescence signal from the tryptophan residues at the interface. The fluorescence yield indicates that tryptophan residues at both interfaces are quenched. In the case of binding in Tris buffer, since only two serines are required to produce the maximal effect, this implies that the positive cooperativity seen between the first and second serine is occurring between binding sites at opposite interfaces. Although a third serine is capable of binding at higher serine concentration, its binding has no additional effect on inhibition or fluorescence. The binding of the fourth serine is completely excluded, possibly due to the domains being so predominantly in the closed position at this point that the last site cannot be adequately populated.

An alternative explanation could be that serine binding at one interface is sufficient to produce a global conformational change in the protein that is able to close the opposite interface in the absence of serine binding to it. In this case, the positive cooperativity for the first two serines would occur with binding of both serines at the same interface. In both cases, maximal inhibition of activity is produced. This alternative is thought to be less likely because of the need to explain how binding of two serines at the same, presumably rigid interface could each produce an approximately equal increase in the degree of inhibition. However, the present data are not sufficient to conclusively distinguish between the two cases.

The binding data measured in phosphate buffer show that three serines bind before the maximal effect is obtained. It follows that binding of three serines must involve binding at both interfaces. This suggests that the phosphate effect is to allow both sites at an interface to become occupied essentially simultaneously by defeating the global confor-

mational change brought about by serine binding to the first site. This suggests that in the presence of phosphate the binding sites are essentially independent of each other and both interfaces must be occupied to produce maximal effect. The Scatchard plot (Figure 4) suggests that some amount of positive cooperativity is retained, but the dissociation constants suggest that it is minimal. In addition, the correlation between the distribution of bound species and the predicted inhibition (Figure 7) suggests that the order of binding changes so that in phosphate one interface is fully occupied (two serines) by the time the second interface binds its first serine.

The data demonstrate that there is a direct relationship between serine binding, conformational change at the regulatory site interfaces, and inhibition of enzyme activity. However, the observation that phosphate does not appear to have an effect on the cooperativity of inhibition suggests that there are two distinct cooperative pathways at work: one between the four serine binding sites, and one between the serine binding sites and the active sites.

The molecular basis for this "phosphate effect" is unknown at the present time. Since a phosphate group is part of the physiological substrate 3-phosphoglycerate, it could be interacting with the active site phosphate locus. Since we know that there is linkage between the serine binding sites and the active sites, phosphate interaction at the active site may show a reciprocal effect on the serine binding sites. In so doing, it may shift the conformational equilibrium of the regulatory domain interface to favor a more open form that results in greater ease of serine binding and that translates into a decrease in cooperative effects. This is presently being investigated further. The correlation between effector binding, inhibition of activity, and conformational change provides insight into the mechanism of regulation of PGDH activity and will undoubtedly be useful for future work designed to reveal the molecular linkage between the two cooperative pathways in PGDH.

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