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Yeast Hexokinase in Solution Exhibits a Large Conformational Change upon Binding Glucose or Glucose 6-Phosphate†

R. C. McDonald, T. A. Steitz, and D. M. Engelman*

ABSTRACT: Using small-angle X-ray scattering from solutions of yeast hexokinase, we have measured the radii of gyration of the monomeric B isozyme and its complexes with sugar substrates. We find that the radius of gyration decreases by 0.95 ± 0.24 Å upon binding glucose and 1.25 ± 0.28 Å upon binding glucose 6-phosphate. This observed reduction in radius of gyration in the presence of glucose is the same as that calculated from the coordinates of the high-resolution crystal structures of native hexokinase B and a glucose complex with hexokinase A. Thus, these measurements suggest that the dramatic closing of the slit between the two lobes of hexokinase observed in the crystal structures (Bennett, W. S., & Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4848–4852) occurs in solution when either glucose or glucose 6-phosphate is bound.

The A isozyme of yeast hexokinase crystallized as a complex with glucose has a conformation that is dramatically different from the conformation of the B isozyme crystallized in the absence of glucose (Bennett & Steitz, 1978; Anderson et al., 1978a). Comparison of the high-resolution structures shows that one lobe of the molecule is rotated by 12° relative to the other lobe, resulting in movements of as much as 9 Å in the polypeptide backbone and closing of the deep cleft between the lobes into which glucose is bound (Bennett & Steitz, 1978). The question arises whether this conformational difference in the two separate crystal structure determinations is due to the difference in hexokinase isozymes used in the two studies or is induced by the binding of the substrate ligand glucose.

Several observations on the enzyme in solution suggest that the binding of glucose induces an enzyme conformational change; however, the nature and magnitude of this change are not indicated. Changes in UV absorption and tryptophan fluorescence are observed upon glucose binding (Danenberg & Cleland, 1975; Hoggett & Kellett, 1976; Peters & Neet, 1978). The affinity of hexokinase for ATP appears to be greater in the presence of glucose substrates and analogues (Danenberg & Cleland, 1975; Kaji & Colowick, 1965), and the low ATPase activity of the enzyme is stimulated by lyxose and xylose (Dela Fuente et al., 1970). Furthermore, it has not been possible to crystallize the A isozyme in the absence of glucose nor the B isozyme as the glucose complex (Bennett & Steitz, 1978; Anderson et al., 1978b), consistent with a glucose-induced structural change in solution. Crystals of the A isozyme disintegrate when glucose is removed, and B isozyme crystals dissolve when exposed to high concentrations of glucose for prolonged periods. These observations combine to suggest a significant conformational effect of glucose on the enzyme.

In order to identify the conformational difference observed in the crystal structures of the liganded and unliganded enzyme with the expected conformational change induced by glucose, we have examined the small-angle X-ray scattering of solutions of yeast hexokinase B in the presence and absence of glucose. This is necessitated by the fact that we cannot crystallize the B isozyme in the presence of glucose or the A isozyme in its absence. We have measured the radii of gyration of yeast hexokinase B and its complexes with glucose and glucose 6-phosphate and have compared these observed radii of gyration with those calculated from the crystal structures. We find that both the crystal structures and the enzyme in solution show a reduction of about 0.9 Å in the radius of gyration upon formation of the glucose complex, and that a similar change is produced on binding of glucose 6-phosphate to the enzyme in solution.

Materials and Methods

Reagents. The yeast hexokinase used in these experiments was the B isozyme obtained from Sigma Chemical Co. (type C-302; products no. H 5875; lot no. 41c-8620-2). The enzyme is prepared by the method of Kaji et al. (1961) and has been proteolytically cleaved by an endogenous yeast protease, resulting in the removal of 11 residues from the amino terminus (Schulze & Colowick, 1969). The proteolyzed enzyme has been shown to be monomeric at basic pH and NaCl concentrations above 0.1 M (Schmidt & Colowick, 1973). Polyacrylamide electrophoretic gels of the Sigma hexokinase both under native conditions and under conditions of denaturation in $NaDodSO_4$ were consistent with the enzyme being 95% pure and lacking the amino-terminal fragment.

The other reagents used were glucose, analytical grade from Mallinckrodt (cat. no. 4912); glucose 6-phosphate, crystalline from Sigma (cat. no. G-7879, lot no. A56-0122); phenylmethanesulfonyl fluoride from Sigma (cat. no. P-7626, cat. no. 100C-1500); sodium azide, practical grade, from Matheson Coleman and Bell; Trizma Base from Sigma; and NaCl, analytical grade, from Fisher.

Preparation of Enzyme for Measurements. The hexokinase obtained from Sigma as an ammonium sulfate precipitate was used without further purification. The ammonium sulfate precipitate was pelleted and dissolved in doubly distilled water to a concentration of about 15 mg of protein/mL of solution. The protein concentrations were calculated based on an $E_{280}^{1\%}$ of 9.49 (Colowick, 1973). The dissolved ammonium sulfate precipitate was divided into two equal volumes. One was dialyzed against 40 mM glucose, 30 mM Tris-HCl, pH 8.5, 0.4 M NaCl, and $\sim 10^{-5}$ M NaN_3 and phenylmethanesulfonyl fluoride; the other was dialyzed against the same buffer without glucose. For the glucose 6-phosphate measurements this procedure was repeated except that all of the dissolved enzyme was dialyzed against 30 mM Tris-HCl, pH 8.5, 0.4 M NaCl, and the NaN_3 and phenylmethanesulfonyl fluoride. Solid glucose 6-phosphate was added to half of this solution to make it 40 mM in glucose 6-phosphate. These enzyme solutions were centrifuged at about 10000*g* just prior to each set of measurements in order to remove any residual solid

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material. The solutions were refrigerated and used within 1 week of preparation.

In order to assess what damage, if any, the X-ray irradiation might be causing, the specific activity of the hexokinase was assayed spectrophotometrically at 25 °C before and after X-ray irradiation in the radius of gyration measurement; no change was found. Also, irradiation produced no alteration in the native gel electrophoresis bands. Repeated measurements of the RG (radius of gyration) over a 24-h period showed that the enzyme does not aggregate at pH 8.5.

Thin-layer chromatography was used to check for traces of glucose in the glucose 6-phosphate material. The solvent system was 2-propanol–pyridine–glacial acetic acid (88:10:40) and the solid phase was silica gel G. No glucose could be found down to the detection limit for the procedure of 0.4%. Given the dissociation constants for glucose and glucose 6-phosphate, the presence of less than 1% glucose in the glucose 6-phosphate would have no significant effect on the scattering experiments.

Scattering Apparatus. Small-angle scattering measurements were made using a Tennelec PSD100 position-sensitive detector. The X-rays were focused from an 8 mm by 50 μm line source produced in a Phillips 1.2-kW fine-focus copper X-ray tube powered by a General Electric XRD6 generator. A single glass mirror bent in a Franks (1958) holder was used to focus the nickel-filtered radiation from the source at the detector plane. The sample was positioned in a thermally controlled holder midway between the mirror and the detector, separated by a 30-cm vacuum path from the detector opening. The line focussed at the detector was 80-μm full width at half maximum, which gave a measured line of approximately 200-μm full width at half maximum when the detector resolution was included. The position linearity of the detector was found to vary by less than 1% over its 8-cm sensitive length, and the efficiency varied by less than 10% over the full width of the detector. A linear region of the detector in which the efficiency varied by less than 1% was used for the small-angle scattering measurements. Small variations in detector properties were compensated by averaging the scattering curves on either side of the beam stop.

Scattering Measurements. A single glass capillary was used to compare the scattering from solutions of hexokinase with that of buffer in a series of measurements at different protein concentrations. Buffer measurements were performed periodically throughout the measurement sequence, and no difference (within statistical error) was observed in comparing curves taken at different times. Similarly, a series of measurements was made on a single protein solution to test for reproducibility in the apparatus. Again, the measured radii of gyration were constant to within the statistical precision of the measurement. Sample temperature was maintained at 20 °C. Data from the position-sensitive detector were stored in a multichannel analyzer and subsequently recorded on magnetic tape. Using a PDP-11/70 computer, the background subtraction was performed, and the data were fitted in the Guinier region to a straight line using a variance-weighted least-squares procedure (Bevington, 1969).

Data Analysis. The radius of gyration was calculated as the slope of a straight line fit to the data in a plot of ln (net intensity) vs. $(2\theta)^2$ where θ is the equivalent Bragg angle. The radius of gyration, RG, is then calculated from eq 1, where

$$\mathrm{RG} = \frac{\lambda\sqrt{3}}{2\pi}\left[\frac{\Delta \ln I}{\Delta(2\theta^2)}\right]^{1/2} \tag{1}$$

I is the net scattered intensity at angle 2θ and λ is the wavelength, taken at 1.54 Å (Guinier, 1939). The statistical error in the calculated radius of gyration was propagated from the counting statistics in the fitting procedure, which also gave the intensity at zero angle, I_0, together with its associated error. Values of I_0/C, where C is the protein concentration, were calculated for each measurement. These values were found to vary by less than 5%, showing that aggregation effects were not present either as a consequence of the addition of glucose or glucose 6-phosphate or as a consequence of variation in the concentration of protein.

In previous experiments, the effect of slit smearing on measured values of the radius of gyration has been explored. Data were collected on solutions of bovine serum albumin, and the resulting curves were desmeared using the method of Glatter (1977). The correction of the observed radius of gyration in the case of this protein was less than 0.1 Å and, since the present measurements are conducted over a comparable range of scattering angles, no correction was deemed necessary for the present measurements. This observation confirms the well-known fact that an extended Gaussian region at small angles is unchanged in slope by slit-height corrections.

The slope of the concentration dependence was determined from the data on hexokinase with no ligands. A modest negative slope was found, which probably arises from interparticle interference effects. The same concentration dependence was used in interpreting the data from all three samples, since no indication of aggregation effects was observed in the measured values of I_0/C. In each case, the data were fit with a line of fixed slope, using a variance-weighted least-squares procedure (Bevington, 1969). The intercept at zero concentration was derived, together with associated errors, and taken as the measured radius of gyration for a particular sample.

Calculated Scattering Curves. Using the atomic coordinates obtained from crystallographic studies, scattering curves and radii of gyration were calculated for hexokinase monomers. Radii of gyration were calculated from the relation

$$\mathrm{RG} = \left(\frac{\sum z_i R_i^2}{\sum z_i}\right)^{1/2} \tag{2}$$

where z_i is the atomic number for atom i and R_i is its distance from the centroid of the atomic distribution in the molecule. Radii were calculated for the monomer with glucose bound using the data from the 3.5-Å resolution structure of hexokinase A (Bennett & Steitz, 1978) and for hexokinase with no substrate bound using data from the 2.1-Å resolution structure of hexokinase B (Anderson et al., 1978).

The continuous scattering curves for hexokinase were calculated using the Debye relationship

$$I(\mathrm{s}) = \sum_i \sum_j z_i^2 + z_j^2 + 2 z_i z_j \frac{\sin(2\pi s R_{ij})}{2\pi s R_{ij}} \tag{3}$$

where $s = (2 \sin\theta)/\lambda$ and R_{ij} is the distance between atoms i and j in the molecule (Debye, 1915). Scattering curves, $I(\mathrm{s})$, were then plotted using the Guinier representation (Guinier, 1939).

Results

The objectives of the experiments presented here are to measure the influence of ligands on the radius of gyration of hexokinase in solution and to compare that influence with the changes calculated from crystal structures of this enzyme. A measureable change in radius of gyration is expected if the properties of monomers of a single isozyme in solution parallel those of the different isozymes in the crystals. Since atom-

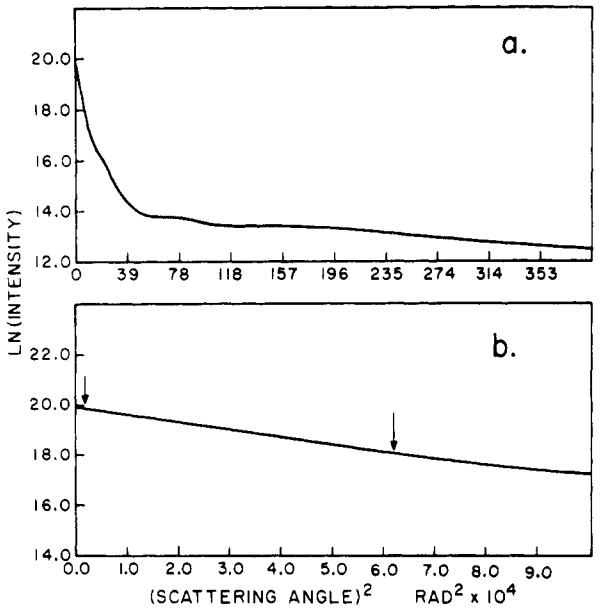

FIGURE 1: Calculated scattering curves for hexokinase monomers in solution. Scattering curves were calculated using the Debye (1915) formula and the atomic positions obtained from crystallographic studies of hexokinase (Bennett & Steitz, 1978; Anderson et al., 1978a). The scattering curves are shown plotted using the Guinier formulation (Guinier, 1939) which permits extraction of the radius of gyration as the slope of the linear portion of the scattering curve near zero angle. (a) The extended scattering curve is shown to a maximum scattering angle corresponding to a Bragg spacing of 8 Å. (b) The very small angle region is shown to a maximum scattering angle corresponding to 50-Å equivalent Bragg spacing. The arrows indicate the region chosen for measurement in the experimental determination of radii of gyration used in this paper. Note that the linear approximation is valid for this molecule over a fairly large range, which permits a precise determination of the radius of gyration from small angle measurements.

ic-resolution structures are available as the basis for theoretical calculation, the interpretation of small-angle scattering curves is placed on a firm basis.

Using the atomic coordinates from the crystal structures, scattering curves and values for the radius of gyration were calculated. Figure 1a shows the extended scattering curve to a resolution of 8 Å for a solution of hexokinase monomers. A sharp decline in the intensity curve is seen at small angles, which then levels out in a slowly rippling function. An expansion of the very small-angle scattering region is shown in Figure 1b. Represented on a Guinier plot, the scattering curve is linear over a wide region. This permits a definitive choice of the region to be taken in subsequent analyses for the radius of gyration using experimental data. The region chosen is indicated by the arrows in Figure 1b.

Calculated radii of gyration were obtained from atomic coordinates using eq 2. By use of coordinates of the B isozyme of hexokinase in the absence of substrate or substrate analogues (Anderson et al., 1978a,b), a value of 23.66 Å is calculated for the radius of gyration. By use of the data from the A isozyme crystallized in the presence of glucose (Bennett & Steitz, 1978), a radius of gyration of 22.76 Å is obtained, giving a net change in the radius of gyration of 0.90 Å as the consequence of the conformational change. No calculation for hexokinase in the presence of glucose 6-phosphate is possible, since no crystals of the enzyme complexed with this ligand have been grown.

Small deviations from the observed radius of gyration are to be expected in carrying out this calculation. An obvious source of error is that approximately 300 of the expected 3600

Table I: Influence of Ligands on the Radius of Gyration of Hexokinase

sample	predicted RG (A)	predicted difference (A)	measured RG (A)	measured difference (A)
hexokinase with glucose	22.76	0.90	23.78 ± 0.14	0.95 ± 0.24
hexokinase	23.66	–	24.73 ± 0.19	1.25 ± 0.28
hexokinase with glucose 6-phosphate	–		23.48 ± 0.20	

atoms are not included in the model derived from the high-resolution refinement (Anderson et al., 1978a). The atoms which are unaccounted for are most likely to be found at the surface of the enzyme, and their omission can be expected to result in a slight reduction in the calculated radius of gyration. An additional difficulty is that hydrogen atoms are not observed and have been omitted from the calculation. Another reason that the observed radius may differ from the calculated radius is that the experimental observation is carried out in the presence of solvent, whereas the calculation gives the radius of gyration at infinite contrast. If the distribution of electron density is not uniform through the protein, as would be expected, a reduction in the contrast due to the presence of solvent could influence the radius of gyration. This influence is strongest when the average electron density of the protein is close to that of the solvent. In the present case, the average contrast is sufficiently high so that this influence should be small. These qualifications to the present calculation should not affect the calculated difference between the two conformations of hexokinase by more than 5% (and probably much less).

Scattering curves for hexokinase solutions were measured in a concentration series. Examples of the measured curves are shown in Figures 2a,b. The data which are shown cover the angular range defined in Figure 1b and are fitted to a straight line using a variance-weighted least-squares procedure (Bevington, 1969). Radii of gyration are calculated from the slope of each line using eq 1, and errors associated with the measured values are propagated from the counting statistics. The fit to the data also provides a measured value for the intensity at zero angle. Scaled to protein concentration, the scatter at zero angle permits assessment of possible aggregation in the sample. Four radius of gyration measurements were rejected because the scaled value of the forward scatter was significantly different from that for the other samples in the run. Additionally, one measured value was rejected on the grounds that it lay more than three standard deviations from the line determined by all other measurements in the series.

The concentration dependence of the measured values of the radius of gyration was determined using the data for hexokinase in the absence of ligands. A least-squares fit to these data gave a modest negative slope, which is expected on the basis of interparticle interference effects. Since the forward-scatter measurement could be used to reject the presence of aggregation effects, the same slope was used to fit the concentration series for each of the three classes of experimental samples.

The observed radii of gyration together with associated errors are shown in Figure 3. The lines fit to these data using a variance-weighted least-squares procedure were used to obtain values for the radius of gyration at zero concentration in each case together with associated statistical errors. Table

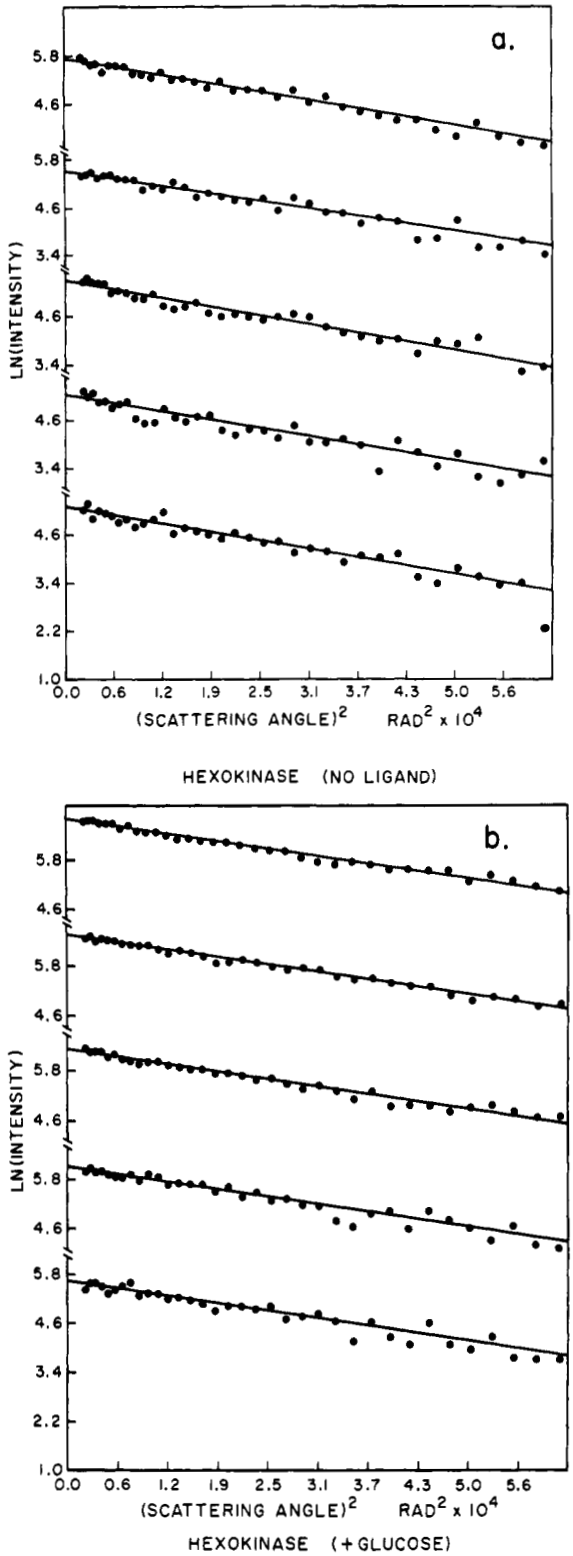

FIGURE 2: Experimental determination of radii of gyration. Examples of data used in the determination of radii of gyration for hexokinase are shown. In each case the data are represented on a Guinier plot, and the line which is shown is the variance-weighted least-squares fit to the experimental data points. The region taken for analysis corresponds to the indicated portion of the scattering curve in Figure 1. (a) Scattering curves of hexokinase in the absence of any ligand are shown at a series of enzyme concentrations. From top to bottom the concentrations used were 14.6, 9.73, 7.3, 5.9, and 4.9 mg/mL. (b) A series of measurements of the radius of gyration of hexokinase in the presence of glucose is shown at different enzyme concentrations. From top to bottom the concentrations used were 11.1, 10.2, 9.0, 7.5, and 5.6 mg/mL. In each case the slope of the line is related to the radius of gyration by the Guinier relationship (eq 1).

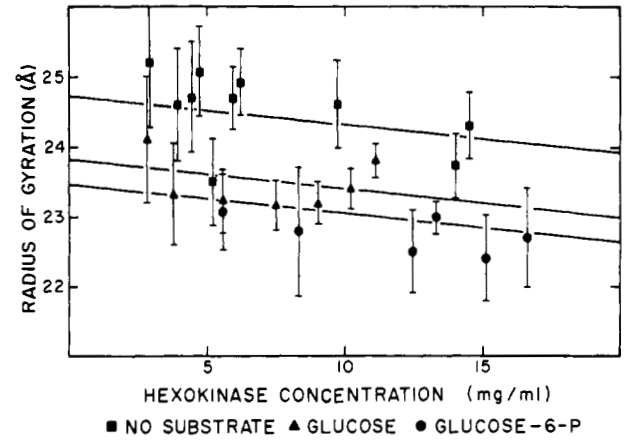

FIGURE 3: Summary of radius of gyration measurements for hexokinase in the presence of various ligands. Values for the radius of gyration are shown for measurements of the type described in Figure 2. The concentration dependence of the scattering was determined from the best data set (no substrate), and the same slope was used in fitting each of the data sets. The variance-weighted least-squares fit is shown for the cases of (■) no substrate, (▲) glucose, and (●) glucose 6-phosphate. The values extrapolated to zero concentration are given, together with associated errors, in Table I. They show a significant change in the radius of gyration between hexokinase in the absence of substrates and hexokinase in the presence of either glucose or glucose 6-phosphate. This difference is of the same magnitude predicted on the basis of the crystal data and confirms the existence of a large conformational change of hexokinase on binding of glucose.

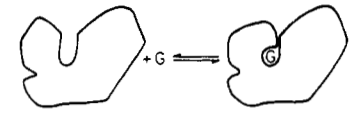

FIGURE 4: A drawing showing the projected outline of the actual crystal structures of the unliganded hexokinase on the left and the liganded hexokinase on the right (Bennett & Steitz, 1978; Anderson et al., 1978a). In the absence of glucose the enzyme has an open structure containing a very deep slit, while in the presence of glucose the conformation changes and has a closed structure (Anderson et al., 1978a).

I gives the measured values for the radii of gyration of hexokinase alone, hexokinase with glucose, and hexokinase with glucose 6-phosphate. A clear difference exists between the enzyme alone and the enzyme in the presence of gluocse or glucose 6-phosphate, and the change with glucose is comparable in magnitude and direction with that calculated from the two crystal structures. The difference between the structures with glucose and glucose 6-phosphate is not statistically significant.

Discussion

The radius of gyration measurements presented here show that the large conformational difference observed between the structures of the A isozyme of hexokinase crystallized with glucose and the B isozyme crystallized without glucose (Figure 4) results from the binding of glucose. The difference in the radius of gyration between the open and closed conformations calculated from these two crystal structures (0.9 Å) is the same as the measured decrease in the radius of gyration of the monomeric B isozyme in solution observed to occur upon binding glucose (Table I). Attempts to use small-angle X-ray scattering measurements to demonstrate that glucose also reduces the radius of gyration of the A isozyme have thus far been frustrated by the tendency of this isozyme to aggregate at the high protein concentrations required (Pickover & Steitz, unpublished observations). Although it is formally a possibility that in solution there is some glucose-induced conformational

change other than the one observed in the crystal structures which fortuitously reduces the radius of gyration by 0.9 Å, we view this as unlikely.

Since the glucose-induced conformational change is sufficiently large to be measured by this solution technique, it is possible to examine the effect of other ligands on the enzyme's structure to see whether the open or closed enzyme structure (or perhaps a third structure) is favored. For example, does the product sugar, glucose 6-phosphate, favor the open or closed structure? The necessity for glucose 6-phosphate to dissociate from the enzyme more rapidly than glucose does (Rose et al., 1974) suggested that this product complex might be in the open conformation. However, the measured radius of gyration clearly shows that the enzyme is also in the closed conformation when glucose 6-phosphate is bound. Therefore, it seems likely that the faster rate of dissociation of glucose 6-phosphate results from an increased rate of the conformational change in the glucose 6-phosphate complex as compared with the glucose complex.

Small-angle X-ray scattering appears to be an extremely useful technique for correlating the results of various enzyme crystal structures with the enzyme's behavior in solution. The radius of gyration measurements are quicker than solving another crystal structure and have the added benefit of providing assurance that the crystal structure of a particular ligation state of a protein represents the predominant conformational species in solution. Conversely, radius of gyration measurements in the absence of the crystal structure data would be less enlightening; they would simply add to the long list of techniques which provide evidence for conformational changes with little indication of their nature or function. The fact that one can use the atomic positions from the crystal structures to predict radii of gyration and to calculate theoretical scattering curves greatly enhances the power of the small-angle scattering method. It is only when the proposition being tested is well defined and constrained by a large amount of other information that small-angle measurements can be decisive.

It might well prove useful to use radius of gyration measurements to indicate which protein–ligand complexes might be of particular interest for crystallographic study. If the binding of a ligand produces a substantial change in the measured radius of gyration, then it is likely that at least two crystal structures will be required. Diffusion of ligands into existing crystals is not necessarily sufficient, since crystal lattice forces may be strong enough to mask ligand-induced conformational changes which occur in solution (Blow & Steitz, 1970). In the case of hexokinase, for example, low concentrations of glucose diffused into hexokinase B crystals for short times (less than 12 h) allow the glucose to bind without producing the full conformational change or destroying the crystal, though higher concentrations and longer soak times disrupt the crystals (Anderson et al., 1978b). Since, in general, proteins are not crystallized and their structures determined both in the presence and in the absence of relevant ligands, the number of proteins that show substantial ligand-induced conformational changes may be rather larger than the current crystallographic data suggest.

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