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Effect of Macromolecular Crowding upon the Structure and Function of an Enzyme: Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The specific activity of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPD) has been measured as a function of GAPD concentration in the absence and presence of 18 g/dL ribonuclease A. The specific activity of GAPD at fixed concentration has been measured as a function of the concentration of added ribonuclease A, β-lactoglobulin, bovine serum albumin, and poly(ethylene glycol) (M_r 20000) at additive concentrations of up to 30 g/dL. All of the data may be semiquantitatively accounted for by a simple model based upon the following qualitative assumptions: (1) Under the conditions of the reported experiments, GAPD exists primarily as an equilibrium mixture of monomers and tetramers of GAPD subunits. (2) The monomers have a much larger specific activity than do the tetramers. (3) The addition of high concentrations of unrelated globular proteins does not affect the activity of either monomer or tetramer but does promote the formation of tetramer due to space-filling properties of the added species, as proposed by Minton [Minton, A. P. (1981) *Biopolymers* (in press)].

Studies of enzyme structure and function in solution are traditionally carried out in solutions which are as dilute in protein as accurate measurement will allow. Measurements of the dependence of a measured property upon protein concentration are most frequently employed as a means of extrapolation to infinite dilution. The properties thus measured are characteristic of an isolated enzyme molecule in a bath of solvent containing salts, substrates, and cofactors as required. Such an environment differs in one very important respect from the fluid medium in which an enzyme ordinarily performs its biological task. Such a medium may indeed be dilute in the particular enzyme of interest but will contain a large variety of other mobile macromolecules, which, taken together, occupy a substantial fraction of the total volume. Such a medium will be described as *volume occupied* or *crowded*.

The effect of volume occupancy upon the thermodynamic activity of globular proteins in solution has been the subject of several studies in this laboratory (Minton, 1977, 1980; Ross & Minton, 1977, 1979; Ross et al., 1978). Recent calculations suggest that crowding may have a substantial effect upon the structure and catalytic activity of an enzyme which is itself present at very low concentrations. Several possible mechanisms for such an effect have been advanced (Minton, 1981), among them the following. Consider an enzyme which may exist in more than one state of self-association (e.g., monomer, dimer, trimer, . . .). If the catalytic activity of the enzyme varies with the degree of self-association and then if crowding alters the average degree of self-association, it will also alter the average catalytic activity of the enzyme.

The study presented here was undertaken in order to answer two questions: (1) Can crowding affect the catalytic activity of an enzyme by the mechanism outlined? (2) If so, how well can the phenomenon be described by the approximate theory of Minton (1981)?

The enzyme D-glyceraldehyde-3-phosphate dehydrogenase (GAPD)[1] catalyzes the reaction

$$\text{D-glyceraldehyde 3-phosphate} + NAD^+ + P_i \rightleftharpoons \text{1,3-diphosphoglycerate} + NADH + H^+ \quad (1)$$

GAPD consists of a tetramer of identical M_r 36000 subunits, which can reversibly dissociate to dimers and/or monomers, depending upon experimental conditions (Hoagland & Teller, 1969; Constantinides & Deal, 1969; Lakatos et al., 1972; Lakatos & Zavodszky, 1976). The specific activity of GAPD has been shown to vary with GAPD concentration (Jancsik et al., 1979) in a manner which suggests that the specific activity of the subunit depends upon the degree of self-asso-

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[1] Abbreviations used: GAPD, glyceraldehyde-3-phosphate dehydrogenase; GAP, 3-phospho-DL-glyceraldehyde (or DL-glyceraldehyde 3-phosphate); βLG, β-lactoglobulin, RNase, ribonuclease A; PEG, poly(ethylene glycol); Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

ciation. For this reason, GAPD was selected as an enzyme which might exhibit the predicted effect of crowding.

The basic theory of the effect of crowding upon self-association equilibria (Minton, 1981) is summarized below. Next, experimental methods are described and results presented. Finally, it is shown that the data may be semiquantitatively described by a simple model in which volume occupancy alters the equilibrium between monomers and tetramers of GAPD subunits without affecting the specific activity of either species.

Theory

Effect of Crowding upon the Self-Association of a Dilute Protein. A general treatment of the effect of excluded volume upon self-association equilibria has been presented by Minton (1981). Here we summarize results which are applicable to the interpretation of experimental data to be presented in the following section.

Consider the following self-association equilibrium of a globular protein in solution:

$$nP_1 \rightleftharpoons P_n \tag{2}$$

The equilibrium constant for this reaction is defined as

$$K_0 \equiv (a_n/a_1^n) = (\gamma_n/\gamma_1^n)(c_n/c_1^n) \tag{3}$$

where a_i, γ_i, and c_i are respectively the thermodynamic activity, activity coefficient, and molar concentration of i-mer. We define the apparent equilibrium constant

$$K_c \equiv c_n/c_1^n = K_0\Gamma \tag{4}$$

where

$$\Gamma \equiv \gamma_1^n/\gamma_n$$

To a highly dilute solution of P we add a second globular protein P′ which is presumed not to bind or otherwise specifically interact with P. The free-energy change associated with the formation of 1 mol of P_n from n mol of P_1 in the presence of P′ is presumed to be the same as in the absence of P′, with one exception. Since molecules of P_1, P_n, and P′ all have finite volumes and cannot intersect each other, the number of permissible ways of placing a given number of molecules of P′ around n separate molecules of P_1 is different than the number of permissible ways of placing the same number of molecules of P′ around one molecule of P_n. The free-energy change accompanying the self-association of P in the presence of P′ will thus contain an additional entropic contribution due to the concomitant alteration of the spatial distribution of P′ molecules in the solution, and Γ will consequently differ from unity.

For an approximate calculation of the effect of P′ upon the value of Γ, several simplifying assumptions are introduced:

(1) Protein molecules in solution do not interact significantly at distances greater than intermolecular contact distances. This assumption is realistic for protein solutions of moderate ionic strength at pH values not far removed from the isoelectric point (Ross & Minton, 1977).

(2) Under these conditions, the protein solution may be formally treated as an equivalent "gas" of hard particles corresponding to each protein solute species (MacMillan & Mayer, 1945). Calculation of protein activity coefficients is thus performed by utilizing methods developed for the study of hard particle fluids. In this fashion, the thermodynamic activity of proteins in solution may be semiquantitatively calculated at concentrations exceeding 30 g/dL if equivalent particles are chosen which resemble the corresponding protein molecule in mass, size, and gross shape (Ross & Minton, 1977, 1979; Ross et al., 1978).

(3) The activity coefficient of a particular species of hard particle in a mixture of hard particles of different sizes and the same shape may be calculated approximately by using the scaled particle theory of Lebowitz et al. (1965) as generalized by Gibbons (1969). The quantitative expressions utilized are presented in Appendix I of Minton (1981).

In general, the activity coefficient of each protein species present in solution is a function of the size, shape, and concentration of all protein species present. It is found, however, that the activity coefficient of a species which is sufficiently dilute (occupying less than about 1% of total solution volume) is independent of its own concentration and depends only upon its own size and shape and the size, shape, and concentration of other species occupying more than about 1% of total solution volume. In the present instance, the activity coefficient γ_i will be a function of the size and shape of i-mer and the size, shape, and concentration of P′.

The activity coefficient of species j in a hard particle fluid of given composition and density is a function of W_j, the amount of free energy required to create a "hole" in the fluid (an element of volume which is free of any part of another particle) large enough to accommodate the presence of an additional particle of species j (Lebowitz et al., 1965):

$$\gamma_j = \exp(W_j/RT) \tag{5}$$

where R is the molar gas constant and T is the absolute temperature. W_j and, hence, γ_j increase as the number density of particles of fixed size increases. The nonideal correction factor Γ may thus be perceived as a measure of the free-energy change ΔW associated with the coalescence of n holes of volume v to form a single hole of volume nv:

$$\Delta W \equiv W_n - nW_1 \tag{6}$$

$$\Gamma = \exp(-\Delta W/RT) \tag{7}$$

It may be shown (Minton, 1981) that while the free energy required to create a hole of volume nv is larger than that required to create a hole of volume v (as expected), it is less than that required to create n holes of volume v. ΔW is negative, and the magnitude of ΔW increases with increasing fluid density. Hence, as P′ is added to a solution of P at fixed concentration, Γ will increase, and the equilibrium between the monomer and n-mer of P is shifted in a direction favoring the n-mer.

Materials and Methods

Grade III yeast NAD, rabbit muscle GAPD, 3-phospho-DL-glyceraldehyde (GAP), bovine β-lactoglobulin (βLG), bovine pancreatic ribonuclease A (RNase), L-cysteine, and poly(ethylene glycol) (M_r 20000) (PEG-20000) were obtained from Sigma Chemical Co. and used without further purification.[2] Crystallized bovine serum albumin (BSA) was obtained from Pentex-Miles Laboratories, and inorganic compounds were commercial analytical grade reagents.

The specific activity of the GAPD was measured at 25 °C in solutions of the following (final) composition: 0.1 M Tris-HCl, 0.017 M Na_2HAsO_3, 0.02 M NaF, 3.33×10^{-3} M L-cysteine, 3.33×10^{-3} M NAD, and 1×10^{-4} M GAP. The

[2] It was found in initial experiments that the GAPD-catalyzed reaction was prematurely terminated in the presence of RNase. We determined that this problem could be avoided by using RNase solutions which had been pretreated by immersion in a flask of boiling water for 1–2 min. No precipitation or change in the UV absorbance of RNase solutions thus treated was observed. Presumably heat treatment of the RNase solution deactivates trace contaminants which interfere with the enzyme-catalyzed reaction.

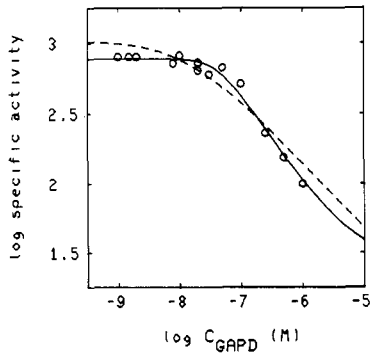

FIGURE 1: Dependence of specific activity of GAPD upon GAPD concentration in the absence of macromolecular additive. (Points) Data. (Dashed line) Best fit of monomer–dimer model (eq 9 and 10) with $\alpha_1 = 1038$, $\alpha_2 = 1.86$, and $\log K^0_{12} = 7.39$. (Solid line) Best fit of monomer–tetramer model (eq 9 and 12) with $\alpha_1 = 773$, $\alpha_4 = 23.9$, and $\log K^0_{14} = 21.16$.

concentration of GAPD was varied over the range 1×10^{-9}–5×10^{-6} M subunit. Other protein or PEG-20000 was present, when indicated, at weight concentrations of up to 30 g/dL. The pH was adjusted to 7.5 after addition of all components except the substrate GAP, which was subsequently added to initiate the reaction.

GAPD activity was assayed by measuring the rate of NADH production in reaction 1. The concentration of NADH formed was determined as a function of time from the absorbance at 340 nm and the molar extinction coefficient $\epsilon^{1cm}_{340} = 6220\ M^{-1}$. Reaction rates at GAPD concentrations of up to 2×10^{-8} M subunit were measured in a thermostated Cary 14 spectrophotometer. At higher GAPD concentrations, reaction rates were measured in a Durrum stopped-flow spectrophotometer. The specific activity was calculated by using the relation

$$\alpha\left(\frac{\text{mol of NADH}}{\text{mol of GAPD} \times \text{min}}\right) = \frac{1}{6220 c_{GAPD}} = \Delta A^{1cm}_{340}/\text{min} \quad (8)$$

where ΔA^{1cm}_{340}/min is the slope of the measured dependence of A^{1cm}_{340} upon time during the initial (linear) phase of the reaction and corresponds to the maximum reaction velocity. Analysis of results obtained in replicate experiments indicates that the standard deviation of measurement is approximately 10% of the measured value.

Results and Discussion

Dependence of Enzyme Activity upon Enzyme Concentration. The specific activity of GAPD in the absence of macromolecular additive is plotted as a function of GAPD concentration in Figure 1. The salient features of this plot are (1) the independence of specific activity upon enzyme concentration in the limit of low enzyme concentration and (2) the roughly 10-fold decrease in specific activity associated with a 100-fold increase in enzyme concentration. In both of these respects, the data presented here qualitatively resemble those of Jancsik et al. (1979), which were obtained under comparable but not identical conditions. Moreover, if the 20-fold difference between the substrate concentrations employed in the two sets of experiments is taken into account, the value of the specific activity obtained at limiting low enzyme concentrations in the present study is in good agreement with that reported by Jancsik et al. (1979).

The observation that enzyme activity decreases with increasing enzyme concentration suggests that the catalytic activity of a GAPD subunit varies with the degree of self-association and that the equilibrium average degree of self-association varies with enzyme concentration. We may write

$$\alpha(c_T) = \sum_i f_i(c_T)\alpha_i \quad (9)$$

where c_T is the total molar concentration of subunits, α the average (observed) specific activity of enzyme, f_i the mass fraction of subunit present as i-mer, and α_i the specific activity of i-mer. The simplest models for concentration-dependent self-association applicable to GAPD are the following:

(a) monomer–dimer ($2P_1 \rightleftharpoons P_2$)

$$K_{12} \equiv c_2/c_1^2 \qquad f_1 = c_1/c_T$$
$$c_T = c_1 + 2c_2 \qquad f_2 = 1 - f_1 \quad (10)$$

(b) dimer–tetramer ($2P_2 \rightleftharpoons P_4$)

$$K_{24} \equiv c_4/c_2^2 \qquad f_2 = 2c_2/c_T$$
$$c_T = 2c_2 + 4c_4 \qquad f_4 = 1 - f_2 \quad (11)$$

(c) monomer–tetramer ($4P_1 \rightleftharpoons P_4$)

$$K_{14} \equiv c_4/c_1^4 \qquad f_1 = c_1/c_T$$
$$c_T = c_1 + 4c_4 \qquad f_4 = 1 - f_1 \quad (12)$$

(d) monomer–dimer–tetramer ($2P_1 \rightleftharpoons P_2$; $2P_2 \rightleftharpoons P_4$)

$$K_{12} \equiv c_2/c_1^2 \qquad f_1 = c_1/c_T$$
$$K_{24} \equiv c_4/c_2^2 \qquad f_2 = 2c_2/c_T$$
$$c_T = c_1 + 2c_2 + 4c_4 \qquad f_4 = 1 - f_1 - f_2 \quad (13)$$

Since the affinity of a subunit for substrates and/or cofactors is presumed to vary with the state of self-association, it follows that the values of self-association constants will depend upon the concentrations of free substrates and cofactors (Hoagland & Teller, 1969; Lakatos & Zavodszky, 1976). However, in our experiments, substrate and cofactor concentrations are held constant and in large excess (at least 30-fold) over the highest enzyme concentrations employed. Under these conditions, values of self-association constants will be independent of enzyme concentration.

The monomer–dimer and monomer–tetramer models were fit to the data for the dependence of log α upon log c_T by the method of nonlinear least squares. The best fit of each model is plotted together with the data in Figure 1; the best-fit values of the variable parameters in each model are given in the figure caption. It may be seen that while the monomer–dimer model (and hence the dimer–tetramer model[3]) cannot adequately fit the data, the monomer–tetramer model provides a reasonably good fit with only three adjustable parameters, α_1, α_4, and log K^0_{14}, where K^0_{14} is the association constant for formation of tetramer in the absence of macromolecular additive.

At the present time, the postulated monomer–tetramer equilibrium is neither supported nor contradicted by direct evidence. The sedimentation equilibrium of GAPD in dilute solution has been measured under conditions somewhat different than those employed in the present study (Lakatos & Zavodszky, 1976). To the extent that one can validly compare results obtained at different temperatures and in different buffers at different pH values, both the sedimentation equilibrium data and the model proposed to account for the dependence of enzymatic activity upon GAPD concentration indicate that at concentrations exceeding 10^{-6} M subunit,

[3] The monomer–dimer and dimer–tetramer models are mathematically homologous; for any set of parameter values in the dimer–tetramer model (K_{24}, α_2, and α_4), it is possible to select a corresponding set of parameter values in the monomer–dimer model (K_{12}, α_1, and α_2) which will yield the identical dependence of α upon c_T.

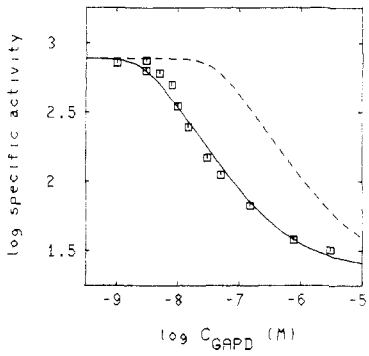

FIGURE 2: Dependence of specific activity of GAPD upon GAPD concentration in the presence of 18 g/dL RNase. (Points) Data. (Solid line) Best fit of monomer–tetramer model (eq 9 and 12) with α_1 and α_4 as in Figure 1 and log K_{14} = 24.61. (Dashed line) Best fit of monomer–tetramer model to data in Figure 1 (no added protein).

GAPD should exist predominantly as a tetramer in the presence of NAD at millimolar concentrations.

The specific activity of GAPD in the presence of 18 g/dL RNase is plotted as a function of GAPD concentration in Figure 2. These data suggest that the limiting values of α at low and high enzyme concentrations may be similar or identical in the presence and absence of 18 g/dL RNase. Accordingly, the monomer–tetramer model was fit to these data with the assumption that α_1 and α_4 are equal to the values obtained from the analysis of data shown in Figure 1. The best fit of this model is plotted in Figure 2 together with the data, and the best-fit value of log K_{14} is given in the figure caption. It may be seen that a reasonably good fit is obtained with only one adjustable parameter.

Some small improvement in the agreement between the calculated and measured dependence of log α upon log c_T in the presence of RNase could probably be obtained if the monomer–dimer–tetramer model were utilized in place of the monomer–tetramer model. However, we did not attempt to fit the monomer–dimer–tetramer model to the data since at least two additional adjustable parameters would have to be introduced. Under these circumstances, an improvement in the fit could not be unambiguously attributed to an improvement in the realism of the model, as it might be due solely to the additional mathematical flexibility conferred upon the model by the presence of additional parameters.

We conclude that our data may be semiquantitatively accounted for by the following hypothesis: Under the conditions of our experiments, GAPD exists predominantly in the form of monomers and/or tetramers of subunits, the proportions of which depend upon the total concentration of enzyme. The specific activity of monomeric subunits is roughly 30 times as great as the specific activity of tetrameric subunits. The addition of 18 g/dL RNase markedly increases the equilibrium association constant for formation of tetramers but does not significantly affect the specific activity of either monomeric and tetrameric subunits.

Hard Particle Model for the Effect of Inert Protein upon the Monomer–Tetramer Association Constant. According to eq 3–7, the dependence of K_{14} upon w_a, the weight concentration of inert additive, will be given by

$$\log K_{14}(w_a) = \log K^0_{14} + \log \Gamma(w_a) \quad (14)$$

$$\log \Gamma(w_a) = [4W_1(w_a) - W_4(w_a)]/(2.303RT) \quad (15)$$

where K^0_{14} is the equilibrium constant in the absence of inert additive.

In order to calculate W_1 and W_4 as functions of w_a, the shapes and relative sizes of monomer, tetramer, and additive

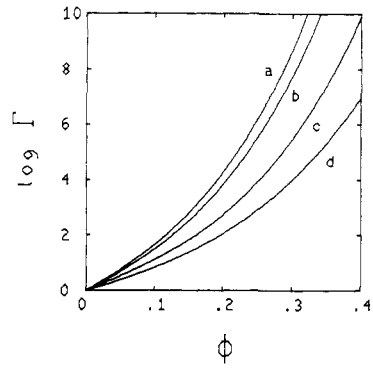

FIGURE 3: Dependence of log Γ upon ϕ, calculated to simulate the effect of adding different proteins as described in text. (Curve a) RNase; (curve b) βLG monomer; (curve c) βLG dimer; (curve d) BSA.

species must be specified. The choice of shape of the equivalent particle is not critical. The conformations of all of the proteins used in the present study are compact, and their gross shapes do not deviate greatly from spherical symmetry (Tanford, 1961, Table 21-1). We have found that the concentration dependence of the thermodynamic activity of hemoglobin, a molecule whose asymmetry is comparable to those studied here, may be accounted for equally well by equivalent hard spheres, cubes, or right circular cylinders with length = diameter (Ross & Minton, 1977). For the present purpose, we shall arbitrarily represent all molecular species by hard cubical particles. The volume of the particle representing monomer is taken to be v_1, and the volume of the particle representing tetramer as $4v_1$. The volume of the particle representing inert additive, v_a, is calculated according to the following approximate relation:

$$v_a = v_1 \frac{M_r(\text{added species})}{M_r(\text{GADP subunit})} \quad (16)$$

The fraction of total volume occupied by additive is defined to be

$$\phi = n_a v_a \quad (17)$$

where n_a is the number density of particles representing additive.

Using the scaled particle theory for hard particle mixtures (Lebowitz et al., 1965) as generalized by Gibbons (1969), we may approximately calculate the values of W_1, W_4, and log Γ as functions of ϕ. So long as v_1 is infinitesimal relative to the unit of volume used to define number density, the calculated values of W_1 and W_4 are independent of the value of v_1 at constant ϕ.[4] Plotted in Figure 3 is the calculated dependence of log Γ upon ϕ for four values of v_a/v_1 corresponding to RNase (M_r 13 000), the monomer and dimer of βLG (M_r 18 000 and 36 000, respectively), and BSA (M_r 70 000).

The fraction of volume occupied by added species, ϕ, is assumed to be proportional to the weight concentration of additive:

$$\phi = V_a w_a \quad (18)$$

The constant of proportionality, V_a, is just the specific volume of the equivalent particle representing additive protein, a quantity which is expected to be slightly greater than the experimentally determined partial specific volume of the

[4] Implicit in this calculation is the assumption that the protein additive does not self-associate at higher concentrations. While none of the additives used in this study have been characterized at high concentration, βLG is known to reversibly self-associate to form dimer at pH 7 (Kelly & Reithel, 1971).

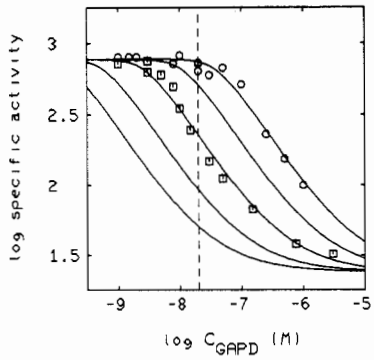

FIGURE 4: Dependence of specific activity of GAPD upon GAPD concentration, calculated for different concentrations of added RNase as described in the text. From right to left, curves are for w_{RNase} = 0, 10, 18, 24, and 28 g/dL. (○) Data for w_{RNase} = 0; (□) data for w_{RNase} = 18 g/dL.

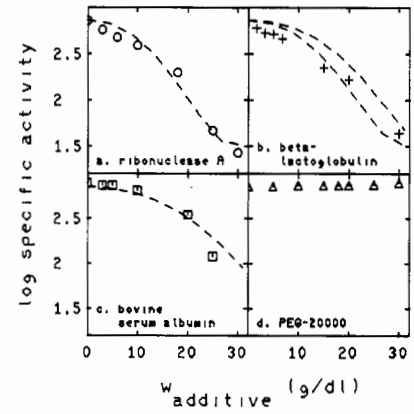

FIGURE 5: Dependence of specific activity of GAPD upon weight concentration of added macromolecular species for $c_{GAPD} = 2 \times 10^{-8}$ M. (Points) Data. Dashed lines calculated as described in text. Added species are (a) RNase, (b) βLG, (c) BSA, and (d) PEG-20 000. Dashed curves in (b) calculated with the assumption that βLG is present entirely as monomer (lower curve) or dimer (upper curve).

protein (Minton & Ross, 1978; Minton, 1980). In an earlier study, we found that the specific volume of the equivalent particle best representing hemoglobin varied between 0.8 and 1.0 mL/g depending upon choice of particle shape (Ross & Minton, 1977).

Calculation of the Effect of Inert Protein upon the Specific Activity of GAPD. The monomer–tetramer/hard particle model may be used to calculate the specific activity of GAPD as a function of the concentrations of GAPD and inert protein by means of the following procedure. Given the values of v_a/v_1, log K^0_{14}, and V_a, the value of log K_{14} may be calculated as a function of w_a by using eq 14 and 15 together with the scaled particle relations presented in Appendix I of Minton (1981). With the value of log K_{14} so obtained and the values of α_1 and α_4, the value of α may be calculated as a function of c_{GAPD} by using eq 9 and 12.

The value of v_a/v_1 is calculated from the molecular weights of additive protein and GAPD by using eq 16. The values of log K^0_{14}, α_1, and α_4 are obtained by fitting the monomer–tetramer model to data obtained in the absence of additive protein, as described above. V_a, which cannot be independently determined, thus constitutes a single adjustable parameter of the monomer–tetramer/hard particle model.

In Figure 4 the dependence of GAPD activity upon GAPD concentration, calculated in the manner described above, is plotted for several values of w_{RNase}. The value of V_a, 0.96 mL/g, was selected to optimize agreement between the calculated curve for w_{RNase} = 18 g/dL and the experimental data which are plotted in the same figure for comparison.

The monomer–tetramer/hard particle model may be used to calculate the dependence of the specific activity of GAPD at fixed enzyme concentration upon the concentration of inert protein. For example, the dependence of GAPD activity at a total GAPD concentration of 2×10^{-8} M subunit upon the concentration of RNase is indicated by the intersections of the calculated curves with the dashed line in Figure 4.

In Figure 5 the specific activity of GAPD at fixed concentration is plotted as a function of the concentration of four inert additive species. PEG-20 000 was selected as a control additive which appears to affect the monomer–oligomer equilibrium of proteins to a much smaller extent than do comparable concentrations of compact globular proteins (Wilf & Minton, 1981).[5] The results shown for PEG-20 000 (Figure 5d) indicate that large changes in the bulk viscosity of the enzyme solution (which increases by a factor of roughly 5.5 at the highest concentrations of PEG-20 000 used here) do not substantially affect the reaction kinetics.

The values of the partial specific volume of all three added proteins differ by less than 3% (Tanford, 1961, Table 21-1). Because of the expected correlation between the values of the partial specific volume and V_a for a particular protein (Minton & Ross, 1978; Minton, 1980), the value of V_a for all three added proteins was set equal to 0.96 mL/g, the value obtained for RNase as described above. The values of v_a/v_1 for RNase, βLG monomer and dimer, and BSA are the same used to calculate the curves shown in Figure 3. By use of these parameter values, the dependence of log α upon w_a was calculated for each of the three added proteins; the calculated dependence is plotted together with the respective data set in Figure 5a–c. The semiquantitative agreement between calculated curves and the data is remarkable in view of the very approximate nature of the model and the absence of any adjustable parameters.

Consideration of Alternative Hypotheses. In principle, the addition of a second protein species to a dilute solution of GAPD may alter the specific activity of the enzyme by mechanisms other than that which we have postulated. Some of these are considered below.

(*1*) *The added species can bind and hence alter the concentration of free substrates and/or cofactors.*

(*2*) *The added species may bind to and induce conformational changes in the enzyme, resulting in altered catalytic activity.* Hypotheses 1 and 2 are inconsistent with the observation that the activity of GAPD in the limit of low enzyme concentration ($<3 \times 10^{-9}$ M) or high enzyme concentration ($>10^{-5}$ M) is not significantly affected by the addition of 18 g/dL RNase.

(*3*) *If encounter rates are rate limiting, the addition of a substance which increases solution viscosity and decreases rates of molecular diffusion may lower observed reaction rates.* This hypothesis is inconsistent with the observation that PEG-20 000, which has an effect on solution viscosity larger than that of an equal weight concentration of any of the protein additives, has no significant effect upon GAPD activity.

(*4*) *The added species may preferentially bind to tetramer, thus increasing K_{14}.* This is the only hypothesis which cannot be ruled out on the basis of the data presented. However, in order to use this mechanism to account for the effects of three different protein additives upon the activity of GAPD, one would have to postulate several binding constants and numbers

[5] It is presumed that the flexible random-coil conformation of PEG in solution permits a certain degree of penetration of protein into the domain of a polymer molecule. Thus the effect of crowding in a solution of PEG or other water-soluble polymer would be smaller than in a solution of globular protein of comparable concentration.

of binding sites which are not independently measurable and, hence, constitute adjustable parameters. Using the hard particle model, we are able to account semiquantitatively for all the data with a single adjustable parameter. Moreover, the physical basis for preferential binding of even one of the added proteins (much less all three) to the tetramer rather than monomer is not apparent.

Conclusion

We have shown that the effect of three globular proteins upon the enzymatic activity of GAPD may be semiquantitatively accounted for by an appealingly simple model which invokes no interaction between molecules of GAPD and the added protein other than mutual impenetrability. We are keenly aware that the model employs several rather strong simplifying assumptions and have attempted to justify each of these approximations, when introduced, on one of the following grounds: (1) The assumption is physically realistic (neglect of long-range electrostatic interactions between protein molecules in solutions of moderate ionic strength). (2) Additional complication would not lead to a significant improvement in agreement between measured and calculated quantities (neglect of GAPD oligomers other than monomer and tetramer; neglect of specific "binding" of additive to GAPD monomer and/or tetramer; oversimplification of particle shape). (3) The assumption has proven useful in analysis of related systems (representation of protein solution by equivalent "gas" of hard particles; calculation of activity coefficients using scaled particle theory). (4) The experimental data required to make the assumption more realistic are lacking (neglect of self-association of additive species at high concentration).

In spite of the approximate nature of the present model, it has proven capable of accounting for a variety of data in a conceptually and mathematically simple manner. We thus believe that the model correctly delineates the two major qualitative effects which dominate the modulation of GAPD activity by added protein at high additive concentrations: the dependence of the specific activity upon the state of subunit self-association and the dependence of the equilibrium average degree of subunit self-association upon the extent of macromolecular crowding in the enzyme-containing solution.

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