Rat Liver Phosphofructokinase: Kinetic and Physiological Ramifications of the Aggregation Behavior†

Gregory D. Reinhart‡ and Henry A. Lardy*

ABSTRACT: The effect of allosteric ligands on the aggregation of rat liver phosphofructokinase (PFK) was examined by measuring the fluorescence polarization of pyrenebutyrate–phosphofructokinase (PB–PFK) as described in the preceding paper. At a protein concentration of 8 μg/mL, near-saturating concentrations of fructose 1,6-bisphosphate, phosphate, or ammonium sulfate stabilize fairly high degrees of polarization whereas AMP and citrate promote polarization values comparable to or below that in the presence of MgATP. However, when each ligand is present in combination with either MgATP or fructose 6-phosphate (F6P), the resulting polarization is very similar to that observed with MgATP or F6P alone, indicating that the influence of MgATP or F6P dominates that of the other ligands. The effect of magnesium adenylyl imidodiphosphate (MgAMPPNP) on the polarization of PB–PFK is very similar to that of MgATP. In the presence of MgAMPPNP and increasing amounts of F6P, the polarization increases, indicating that as F6P binds the aggregation equilibria are shifted toward association. In the presence of MgAMPPNP and low levels of F6P, activators that promote F6P binding also promote aggregation. Activators also promote F6P binding to a small M_r enzyme population with subsequent aggregation and reactivation. These observations are supportive of the hypothesis that F6P binds more favorably to the high M_r aggregate population than it does to the tetramer or smaller populations. Gel filtration of 100000*g* × 60 min supernatant fractions of rat liver homogenates, as well as the ligand effects described, supports the conclusion that a substantial portion of the PFK in the cell exists as a high M_r aggregate. Hence, it is proposed that PFK in vivo binds F6P better than during the highly diluted conditions of kinetic assays in vitro and should therefore be more active at the low F6P concentration found in the cell. This proposal is supported by the finding that specific activity increases with PFK concentration when measured under inhibiting conditions.

Rat liver phosphofructokinase aggregates to high molecular weight species (50 S) even at very low protein concentration in isotonic pH 7 buffer (Reinhart & Lardy, 1980b). The dissociation constant of these polymers is differentially influenced by the two kinetically antagonistic ligands F6P[1] and MgATP; the dissociation constant is lower in the presence of F6P. The enzyme will also spontaneously dissociate to a mixture consisting largely of monomers and dimers when diluted in the absence of ligands other than the K^+ and Mg^{2+} normally present in the buffer. In this form, MgATP promotes aggregation more readily than F6P.

In this paper the influence of allosteric ligands on the aggregation behavior of rat liver PFK is examined, with emphasis placed upon the physiologically important activators FBP, AMP, and P_i. In addition, the opposing influences of MgATP and F6P are further studied by examining the aggregation behavior of the enzyme in the presence of both F6P and the MgATP analogue MgAMPPNP. The net result of these studies suggests that rat liver PFK exists in the cell as a mixture of tetramers and high molecular weight aggregates with the latter representing a significant and probably predominant species. The kinetic consequence of this conclusion is then considered since the aggregate distribution in the cell is therefore quite different from the mostly tetramer distribution present during most kinetic assays.

Once again, the aggregation properties of rat liver PFK are studied by measuring the fluorescence polarization of the pyrenebutyrate-labeled enzyme. The justification for interpreting polarization changes of the modified enzyme as reflecting changes in the aggregation state of the native enzyme has been discussed (Reinhart & Lardy, 1980b).

Materials and Methods

Nucleotides, F6P, FBP, DTT, and coupling enzymes were obtained from the sources indicated in Reinhart & Lardy (1980a,b). In addition, AMPPNP (sodium salt) was purchased from P-L Biochemicals. Rat liver PFK was purified as described (Reinhart & Lardy, 1980a). Pig kidney FBPase was the generous gift of Dr. Giovanna Colombo.

Kinetic assays were performed either at pH 8 under optimal conditions or at pH 7 using the pyruvate kinase–lactate dehydrogenase couple as described previously (Reinhart & Lardy, 1980a) with the exception that pig kidney FBPase was included in the pH 7 assay in a 500-fold excess to PFK activity.

Gel filtration experiments were performed on a Bio-Gel A-5m agarose column measuring 1.4 × 39 cm. Sample size measured 3 mL, and 1-mL fractions were collected; elution was performed at 4 °C.

Rat liver PFK was labeled with pyrenebutyric acid, and polarization experiments were performed at 25 °C as described (Reinhart & Lardy, 1980b).

Homogenization buffer contained 50 mM Tris-HCl, 50 mM NaF, 5 mM DTT, and 1 mM ATP, pH 8.0. Isotonic buffer used in the polarization experiments included 50 mM Mops–KOH, 100 mM KCl, 2 mM DTT, 100 μM EDTA, and 5 mM $MgCl_2$, pH 7.0.

† From the Department of Biochemistry and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received September 6, 1979.* Supported by Grant AM 10334 from the National Institutes of Health.

‡ Present address: Department of Cell Biology, Mayo Clinic, Rochester, MN 55901.

[1] Abbreviations used: F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; P_i, inorganic phosphate; AMPPNP, adenylyl imidodiphosphate; DTT, dithiothreitol; FBPase, fructose 1,6-bisphosphatase; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetate; PFK, phosphofructokinase; PB–PFK, pyrenebutyrate–phosphofructokinase conjugates.

0006-2960/80/0419-1491$01.00/0

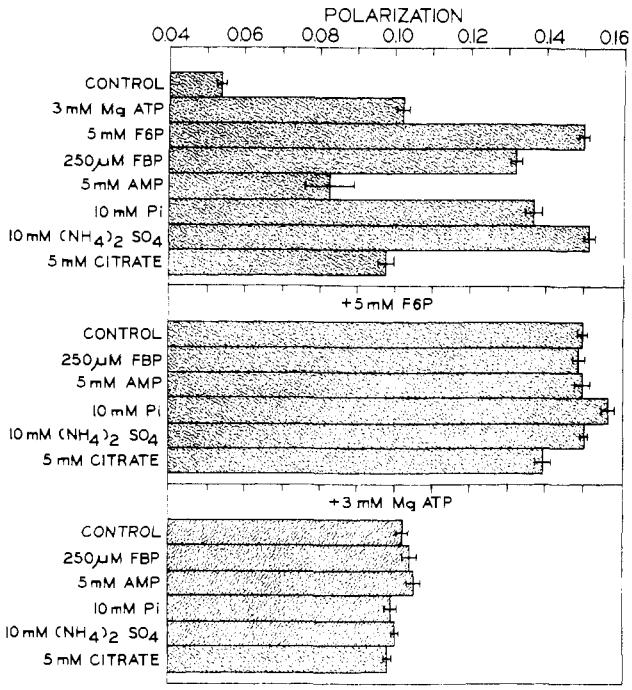

FIGURE 1: Polarization of PB–PFK at 8 μg/mL in the presence of various ligands. Control in the top panel contains isotonic pH 7 buffer containing 5 mM Mg^{2+} as described in the text. Others also contain the ligand indicated. Bottom two panels contain data for ligand plus either F6P or MgATP as indicated. Polarization measurements were made after 5-min incubation at 25 °C where PB–PFK was the last addition. Values are averages from 10 readings with the standard deviation indicated by error bars.

Results and Discussion

The influence of near-saturating levels of several physiologically important ligands on the polarization of PB–PFK at 8 μg/mL can be seen in the top panel of Figure 1. The absence of ligands other than those in the standard isotonic pH 7 buffer results in a very low polarization value (0.054). Addition of 3 mM MgATP or 5 mM F6P, prior to addition of PB–PFK, results in polarization values of 0.102 and 0.150, respectively. As discussed in the preceding paper, these three cases represent a small M_r population, a mostly tetramer population, and a population containing a significant amount of high M_r aggregate, respectively. These polarization values can be compared to the values obtained with several other ligands under otherwise identical conditions. The physiological activators FBP and P_i promote polarization values slightly lower than that of F6P while ammonium sulfate, a nonphysiological but very potent activator, appears to stabilize the same extent of polarization as F6P. The inhibitor citrate, on the other hand, induces a polarization very near that of MgATP. Interestingly, AMP promotes a much lower degree of polarization than do the other activators.

The allosteric ligands do, therefore, have varying influences on the aggregation equilibria of rat liver PFK. These influences can be compared to the results of Lad et al. (1973), who similarly examined the influences of various ligands on the aggregation of rabbit muscle PFK. At a substantially higher protein concentration (0.15 mg/mL) and pH 7, MgATP, F6P, and FBP all stabilized the tetrameric form of the enzyme (Stokes' radius of 65–68 Å). AMP and P_i stabilized an average Stokes' radius slightly smaller than the tetramer (57–61 Å) while citrate significantly destabilized the quaternary structure to a mixture of monomers and dimers (Stokes' radius of 37 Å). Once again, differences in aggregation properties between the liver isozyme and the more frequently studied muscle isozyme are apparent.

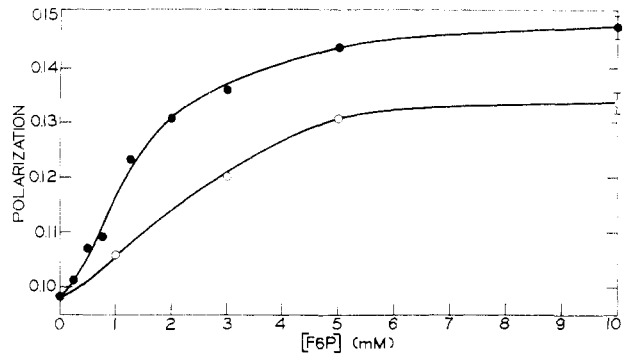

FIGURE 2: Polarization of 8 μg of PB–PFK per mL in the presence of 3 mM MgAMPPNP and varying amounts of F6P. Measurements were made as described in Figure 1. The representative standard deviation is indicated for 10 mM F6P for each curve. (●) PB–PFK was the last addition; (○) F6P was the last addition, 2 min after PB–PFK addition.

The influence of the allosteric ligands in the presence of either substrate is shown in the lower two panels of Figure 1. The polarization value of PB–PFK in the presence of each of the effectors plus 5 mM F6P is very near that for PB–PFK with F6P alone. Similarly, each of the effector ligands in the presence of MgATP has very little if any influence on the polarization compared to MgATP alone. There are two possible explanations for this behavior. One is that the binding of MgATP or F6P interferes with the binding of the effector ligands so that they have little influence in the presence of either substrate. The second possibility is that the influence of F6P or MgATP dominates the influence of the effector ligand with regard to the aspects of protein conformation relevant to its aggregation properties. This in turn could be a consequence of a much larger free energy of interaction of MgATP and F6P with the protein than for the allosteric ligands, or (more likely) the localized conformational influence of MgATP and F6P is in an area more critical to the aggregation properties of the protein than that of the allosteric ligands.

In light of the nature of the kinetic influence of the effector ligands as well as their diversity, it would seem unlikely that both MgATP and F6P would block the interaction of each allosteric ligand with the enzyme. On the contrary, the activator ligands in particular act sympathetically in promoting F6P binding and thus could not reasonably be expected to bind competitively.

The effect that varying F6P concentration, in the presence of 3 mM MgAMPPNP, has on PB–PFK polarization can be seen in Figure 2. AMPPNP is structurally very similar to ATP and yet is not normally a substrate for enzymes that hydrolyze ATP between the β- and γ-phosphates (Yount et al., 1971a,b). Hill & Hammes (1975) have shown that AMPPNP binds to both the active and allosteric nucleotide sites of muscle PFK, although Wolfman et al. (1978) and Pettigrew & Frieden (1979) have concluded that the interaction of AMPPNP with the inhibitor site is not as strong as is the interaction of ATP.

In the absence of F6P, 3 mM MgAMPPNP produces a polarization value (0.098) which is comparable to that in 3 mM MgATP (0.102; see Figure 1). As F6P concentration is increased prior to the dilution of PB–PFK, polarization is increased until at 10 mM F6P the polarization value (0.147) is very close to that observed in the presence of F6P without any nucleotide present (0.150; see Figure 1). When F6P is added after dilution of PB–PFK in the presence of nucleotide,

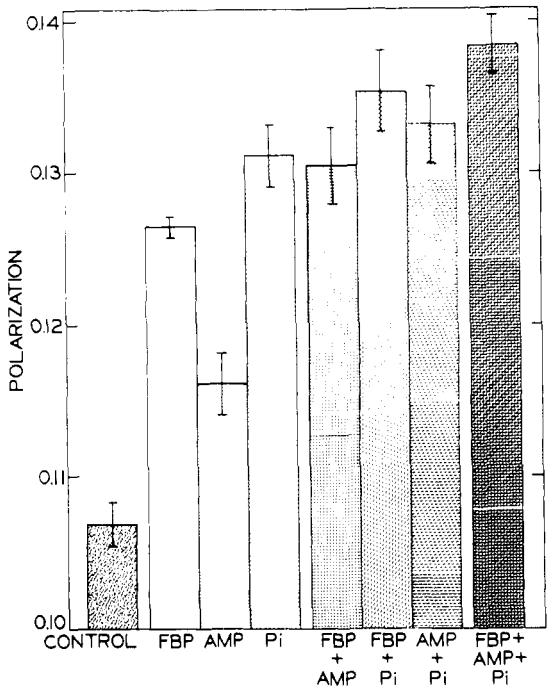

FIGURE 3: Influence of physiological activators on the polarization of PB–PFK at 8 μg/mL in the presence of 3 mM MgAMPPNP and 0.5 mM F6P. Ligand concentrations: 40 μM FBP, 0.5 mM AMP, and 5 mM P_i. PB–PFK was the final addition, and the average ± standard deviation is shown for 10 readings made after 5-min incubation.

the polarization increases only to 0.133 at 10 mM F6P. The possible significance of this result will be discussed below. In either case, the shape of the curves in Figure 2 is noticeably similar to the shape of the kinetic F6P profiles described by Reinhart & Lardy (1980a). Not only do F6P and MgATP act antagonistically in their kinetic influence on the enzyme but they also have opposing influences on the aggregation properties of the protein. Clearly, as F6P binds it is shifting the tetramer–polymer equilibrium established by the nucleotide, in this case MgAMPPNP, toward polymer. Since protein dissociation and ligand dissociation are intimately related (Weber, 1971, 1972, 1975), the likely explanation for this behavior is that the polymeric form of the enzyme has a greater affinity for F6P and/or a lesser affinity for MgATP than does the tetrameric form.

Even though MgATP and F6P dominate the allosteric effector ligands in their influence on the aggregation state of rat liver PFK (Figure 1), the effector ligands do alter the relative affinity of F6P and MgATP as evidenced by the kinetic behavior demonstrated previously (Reinhart & Lardy, 1980a). Consequently, the effector ligands could be expected to alter the aggregation properties of the enzyme in the presence of both MgATP (or MgAMPPNP) and F6P. This possibility is addressed in the experiment described in Figure 3.

At low F6P concentration (0.5 mM) in the presence of 3 mM MgAMPPNP, the polarization of PB–PFK at 8 μg/mL is 0.107, only a small amount greater than the polarization in the presence of MgAMPPNP alone. The polarization under these conditions would be expected to increase if the affinity of the enzyme for F6P were to increase via the addition of activators. This is precisely the result shown in Figure 3. All three physiologically important activators—FBP, AMP, and P_i—increase the polarization of PB–PFK. This is equivalent to decreasing the K_d for F6P in Figure 2 just as was the case in the kinetic experiments. Moreover, the effect is seen with

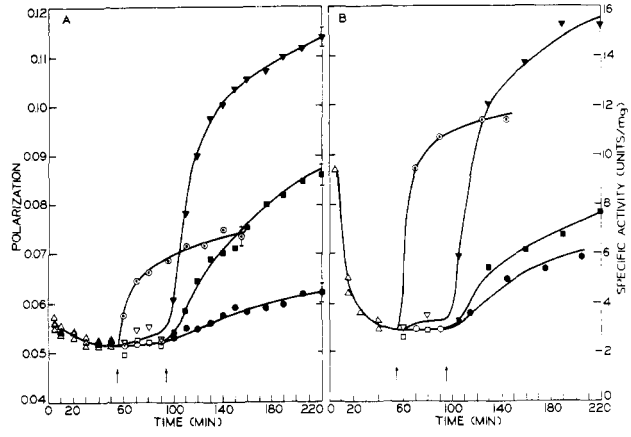

FIGURE 4: Influence of ligands on the polarization and specific activity of a small M_r population of PB–PFK. At time t = 0, PB–PFK was diluted to 8 μg/mL in the absence of addition ligands (△). At t = 55 min (first arrow), either 3 mM MgATP (⊙), 250 μM FBP (▽), 5 mM P_i (□), or 5 mM AMP (○) was added. At t = 95 min (second arrow), 5 mM F6P was added to incubations containing FBP (▼), P_i (■), or AMP (●). Polarizations in panel A are averages of six readings taken over 5 min beginning at the time point indicated. The representative standard deviation is on the last point of each curve. Panel B contains specific activities measured in standard pH 8 assay of a duplicate experiment performed under identical conditions.

the same physiologically optimal concentrations of activators that produced the dramatic kinetic activation in Figure 7 of Reinhart & Lardy (1980a). Combining the activators produces still further increases in polarization, and the polarization in the presence of all three activators is greater than with any two, indicating a contribution from each at these concentrations.

The influence of the allosteric activators on liver PFK aggregation via their ability to promote F6P binding is further demonstrated in Figure 4. It has been shown that F6P does not promote the aggregation of a dissociated population of the enzyme to any significant extent over the course of 3 h after its addition (Reinhart & Lardy, 1980b). It was proposed that this was due to a very low affinity of the low M_r species for F6P. In Figure 4, PB–PFK was allowed to dissociate, and at 55 min a high level of either FBP, P_i, or AMP was added. These ligands have little or no effect by themselves. However, upon subsequent addition of F6P at 95 min (second arrow), the polarization (Figure 4A) and activity (Figure 4B) both increase. FBP shows the greatest effect, with polarization rising from 0.05 to 0.11 and activity increasing nearly fivefold. Polarization with P_i and F6P recovers 0.035 to close to 0.09 while activity rises about 2.5-fold. AMP plus F6P generates an increase in polarization of only about 0.01 while activity is stimulated twofold over 2 h.

The synergistic effect of F6P plus allosteric ligand on the aggregation of the monomer–dimer population to active tetramer or larger species could be explained in two ways. Either the combined effects of F6P and the ligand are necessary to generate the proper protein conformation for aggregation or the influence of F6P is sufficient but the additional ligand is required to promote the F6P binding. The latter explanation would be more consistent with the kinetic role of the allosteric ligands as well as the data presented in Figures 1–3.

It is interesting to note the amount of activity restored, relative to the increase in polarization, as a consequence of the ligand additions shown in Figure 4. As first noted in the preceding paper (Reinhart & Lardy, 1980b), MgATP produces an elevation in both the activity and polarization of a small M_r population of PB–PFK. If these effects are compared to the effects on activity and polarization of P_i and F6P, the

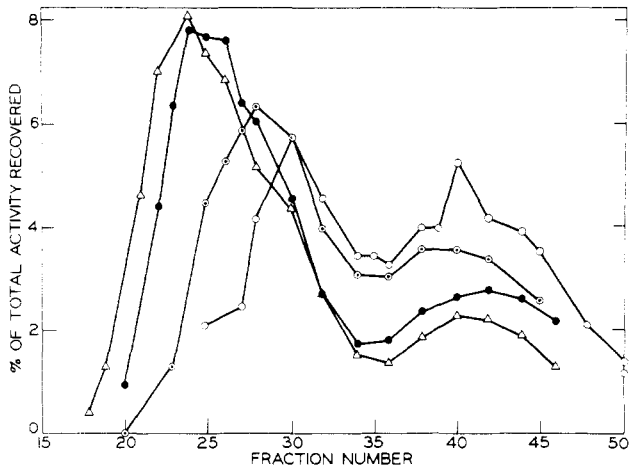

FIGURE 5: Gel filtration behavior of PFK activity in rat liver cytosolic fractions of varying concentration. One-milliliter fractions were collected and assayed at pH 8 under optimal conditions. Fraction 40 is the expected position of the tetrameric enzyme. Heavier species elute at earlier fractions. One gram of liver was homogenized in either 10 (○), 3 (⊙), or 1 mL (●) of homogenization buffer or 1 mL of isotonic pH 7 buffer including 3 mM MgATP and 5 mM KP_i (△).

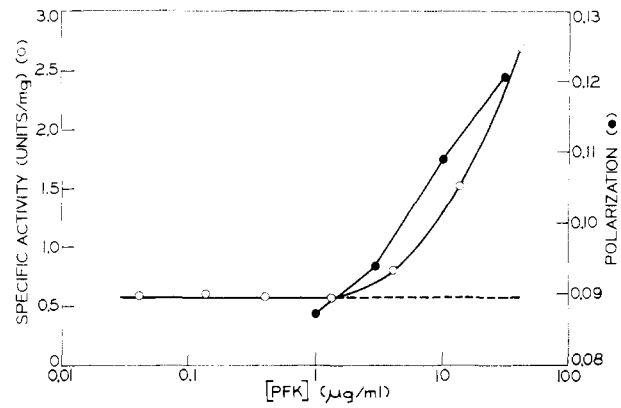

FIGURE 6: Effect of varying PFK concentration on the specific activity as measured at the highly inhibited conditions of 3 mM MgATP and 1 mM F6P at pH 7.0. ADP production was measured via the pyruvate kinase-lactate dehydrogenase coupled assay and FBPase was varied in a constant ratio to the PFK to prevent an increase in the steady-state concentration of FBP. Assays were initiated with the addition of F6P. Total units of FBPase per assay was 500 times the activity of the PFK represented by the straight and dashed lines. Polarization data were obtained from PB-PFK under identical assay conditions except for the exclusion of NADH. Both substrates continued to be recycled (at the expense of PEP) during the course of the measurements. Very low signal to noise ratios prevented us from obtaining polarization readings at lower concentrations.

polarization increase with MgATP is less, yet the activity increase is significantly greater. These data could imply that the average size of the active protein in the presence of P_i plus F6P is larger than that in the presence of MgATP. A similar relationship is apparent for FBP plus F6P compared to MgATP. AMP plus F6P, however, does not produce a relative size as large as does P_i plus F6P since the activity after 220 min is much closer to the P_i plus F6P case than is the polarization. The precise influences of these ligand combinations on the aggregation states are impossible to deduce from these data because of the large number of possible aggregation species involved and the large amount of inactive PFK still present. However, the average size of the aggregate population being formed would appear to be larger in F6P with either P_i or FBP than it is with either MgATP or F6P plus AMP.

The physiological concentration of rat liver PFK is between 20 and 50 μg/mL (Reinhart & Lardy, 1980b). The PB-PFK concentration examined in most of the polarization experiments described has been 7–10 μg/mL, just below the physiological concentration. Consequently, the PFK concentration in the cell is sufficient to allow for the aggregation behavior demonstrated in these experiments. If these results are indicative of the behavior of the enzyme in the cell, one might conclude that (1) liver PFK most likely does not exist extensively as a low M_r mixture since it is continually exposed to stabilizing amounts of ligands and (2) liver PFK exists in an equilibrium mixture of tetramers and higher order aggregates. To investigate this latter conclusion more directly, we performed the following gel filtration experiments.

Livers from fed rats were homogenized at a ratio of 1 g of liver to either 10, 3, or 1 mL of homogenization buffer. The homogenates were spun at 100000*g* for 1 h, and the resulting supernatant solutions were chromatographed on a Bio-Gel A-5m gel filtration column. The results are presented in Figure 5. As the concentration of the cytosolic fraction approaches that of the cell, more of the PFK is present in the high molecular weight fraction. When the homogenization is performed in the more physiological isotonic pH 7 buffer supplemented with 3 mM ATP and 5 mM KP_i at a 1:1 ratio, 80% of the enzyme elutes in the heavier fraction. These data, together with the polarization data described to this point, support the conclusion that rat liver PFK attains a significant, and probably large, degree of high M_r aggregation beyond the tetramer in the cell despite the low naturally occurring concentration of the enzyme.

This conclusion can once again be compared to the behavior of rabbit muscle PFK. Lad et al. (1973) have concluded that the physiological concentration of muscle PFK is on the order of a few tenths of a milligram per milliliter. Though considerably higher than the physiological liver isozyme concentration, the muscle enzyme at this concentration does not readily aggregate beyond the tetramer (Pavelich & Hammes, 1973) even in the presence of various activator ligands (Lad et al., 1973). Consequently, despite similar subunit and tetramer molecular weights, the physiologically important aggregate species may be very different in the liver compared to that in the muscle.

Given the hypothesis that the high M_r aggregate species of rat liver PFK are relevant to the physiological situation, one should then ask what influence, if any, this aggregation might have on the kinetic properties of the enzyme since liver PFK is most dissociated to tetramers at the protein concentration used for the assay (Reinhart & Lardy, 1980b).

A possible answer to this question lies in the observation made previously that the aggregate species may have a greater affinity for F6P than does the tetramer and that the opposite relationship holds for MgATP. The mechanism of the MgATP inhibition as well as the allosteric activation of the ligands examined by Reinhart & Lardy (1980a) revolves around the binding of F6P. MgATP inhibits the binding of F6P while the activators work to remove this inhibition. If the high M_r forms of the enzyme bind F6P better than does the tetramer form, for which these kinetic characteristics were measured, then the aggregation brought about by an increase in protein concentration would work to alleviate apparent MgATP inhibition. The aggregation mechanism would also work to augment the action of the activators since they not only promote binding per se but also promote aggregate formation in the process.

To test this prediction, we varied PFK concentration in a constant ratio to added FBPase under the severely inhibiting assay conditions of 3 mM MgATP and 1 mM F6P at pH 7. These conditions allow for a maximal response to any increase

in affinity of PFK for F6P, as well as assuring that the steady-state concentration of the activator FBP remains constant (McClure, 1969).

The results of this experiment are shown in Figure 6, and, as predicted, the specific activity of the enzyme increases at high concentration. Moreover, PB-PFK incubated under identical conditions shows a significant polarization increase in the same concentration range consistent with an increase in molecular weight.

If this hypothesis is correct, the polarization data for Figure 2 may also explain a kinetic result of Reinhart & Lardy (1980a). In the kinetic experiment, the steady-state K_m for F6P measured when F6P was used to initiate the reaction was higher that that measured when PFK or MgATP addition initiated the reaction. In Figure 2, in the presence of AMPPNP, a comparable procedural change produced a change in polarization throughout the F6P saturation curve. When F6P was added last, the average size was smaller. The reason for this is unknown. However, a higher K_m for the smaller size distribution is predicted by the above hypothesis. It is true that the polarization experiment was performed at a higher enzyme concentration than the kinetic experiment, and we have previously concluded that the enzyme dissociates to the tetramer form in either F6P or MgATP at assay concentration. However, the dissociation in F6P may not be complete, and a small shift in the aggregation state could be responsible for the small shift in the K_m for F6P.

It is easy to postulate a mechanistic explanation for the improved binding of F6P to the aggregate in the presence of MgATP. In many cases protein aggregation is accompanied by an inhibition of enzymatic activity because access of substrates to the active site becomes hindered. Since the poor F6P binding to the tetramer is caused by the binding of MgATP to an allosteric site, it is quite possible that upon aggregation these inhibitor sites become buried and hence MgATP is not as effective in inhibiting the F6P binding to the aggregate.

The physiological ramifications of the increased activity of the aggregate at low F6P concentration may help reconcile the in vitro kinetic behavior of the enzyme with the apparent in vivo activity. The affinity of the enzyme for F6P in the cell must be high and hence MgATP inhibition must to a large extent be alleviated to account for observed glycolytic rates as well as possible "substrate cycling" (Reinhart & Lardy, 1980a). This activity cannot be explained fully by the actions of the most important activators alone. The altered kinetic characteristics of the aggregated enzyme proposed here may explain the in vivo activity.

Little mention has been made in this or the preceding two papers of the phosphorylation of rat liver PFK discovered by Brand & Söling (1975) and its implications for these results. Though no attempt has been made directly to measure the amount of covalently attached P, the observations of Brand & Söling (1975) and Brand et al. (1976) suggest that the PFK used in these studies is fully phosphorylated. Brand et al. (1976) have observed that PFK from fed livers, the source of the enzyme used in our preparations (Reinhart & Lardy, 1980a), is mostly phosphorylated. In addition, any dephosphorylated enzyme present at the time of extraction should have been removed in both the DEAE and gel filtration steps of the purification, based upon the altered chromatographic properties of the dephosphorylated form described by Brand & Söling (1975). Further evidence is given by the fact that the specific activity of our PFK preparations was as high as any reported for this isozyme (Reinhart & Lardy, 1980a).

Likewise, the specific phosphatase and kinase necessary to effect a change in the phosphorylation state of rat liver PFK should not have survived the purification (Brand & Söling, 1975, 1979). Consequently, the ligand-induced changes in aggregation behavior described in these studies are not the result of a change in phosphorylation. This conclusion is supported by the observation of similar results when MgAMPPNP is substituted for MgATP, particularly in the association of the monomer–dimer population (data not shown).

It is likely that this phosphorylation mechanism affects the aggregation properties of rat liver PFK, not only at the monomer–tetramer level as described by Brand & Söling (1975) but also at the tetramer–polymer level discussed here as well. However, since only the phosphorylated form of the enzyme is active (Brand & Söling, 1975), we feel that the aggregation behavior of this form of the enzyme, and the kinetic ramifications of this aggregation, has particular relevance to the regulation of rat liver PFK in vivo.

Added in Proof

It has recently come to our attention that Hofer (1971) has proposed a similar influence of the high molecular weight aggregation on the kinetics of rabbit muscle PFK.

References

Brand, I. A., & Söling, H. D. (1975) *FEBS Lett. 57*, 163–168.

Brand, I. A., & Söling, H. D. (1979) *Proc. Int. Congr. Biochem., 11th*, 306 (Abstract No. 04-5-S130).

Brand, I. A., Müller, M. K., Unger, C., & Söling, H. D. (1976) *FEBS Lett. 68*, 271–274.

Hill, D. E., & Hammes, G. G. (1975) *Biochemistry 14*, 203–213.

Hofer, H. W. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 997–1004.

Lad, P. M., Hill, D. E., & Hammes, G. G. (1973) *Biochemistry 12*, 4303–4309.

McClure, W. R. (1969) *Biochemistry 8*, 2782–2786.

Pavelich, M. J., & Hammes, G. G. (1973) *Biochemistry 12*, 1408–1414.

Pettigrew, D. W., & Frieden, C. (1979) *J. Biol. Chem. 254*, 1887–1895.

Reinhart, G. D., & Lardy, H. A. (1980a) *Biochemistry* (first paper of three in this issue).

Reinhart, G. D., & Lardy, H. A. (1980b) *Biochemistry* (second paper of three in this issue).

Weber, G. (1971) *Horm. Steroids, Proc. Int. Congr., 3rd, 1970*, 58–65.

Weber, G. (1972) *Biochemistry 11*, 864–878.

Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83.

Wolfman, N. M., Thompson, W. R., & Hammes, G. G. (1978) *Biochemistry 17*, 1813–1817.

Yount, R. G., Babcock, D., Ballantyne, W., & Ojala, D. (1971a) *Biochemistry 10*, 2484–2489.

Yount, R. G., Ojala, D., & Babcock, D. (1971b) *Biochemistry 10*, 2490–2496.