Activation of Rabbit Muscle Phosphofructokinase by F-Actin and Reconstituted Thin Filaments†

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ABSTRACT: Striking effects of F-actin and the reconstituted thin filament of muscle on the catalytic activity of rabbit muscle phosphofructokinase are demonstrated through direct measurements of enzymatic activity by using the pH stat. The addition of F-actin to solutions of phosphofructokinase at low ionic strength (10 mM KCl and 5 mM $MgCl_2$) partially reverses the inhibition of the enzyme seen at high ATP concentrations and increases the apparent affinity of the enzyme for fructose 6-phosphate with slight effect on V_{max}. F-Actin augments the activation of the enzyme obtained with AMP and partially counters the inhibition obtained with citrate. The *maximum* effect in the reversal of ATP inhibition is about the same for combinations of either F-actin or the thin filament with AMP as it is for AMP alone. In general, the effect of F-actin on the catalytic activity of phosphofructokinase is larger than that of the thin filament. The activation of phosphofructokinase by F-actin persists at physiological ionic strength.

Phosphofructokinase is considered to be the controlling enzyme in the glycolytic pathway of yeast and mammals (Hofmann, 1978). As such it is subject to close metabolic regulation. The role of substrates and other metabolites as allosteric effectors of phosphofructokinase is well recognized (Bloxham & Lardy, 1973; Hofmann, 1978; Uyeda, 1979). Accumulating evidence shows that macromolecular interactions also affect the catalytic activity of the enzyme. In the case of rabbit muscle phosphofructokinase, experiments have shown that the catalytic activity (Hofer, 1970; Lad et al., 1973) and substrate binding (Hill & Hammes, 1975) are affected by the self-association of the enzyme. Dunaway & Segal (1974, 1976) have discovered a polypeptide phosphofructokinase stabilizing factor in liver and kidney. Adsorption to the inner surface of erythrocyte membranes alters the catalytic properties of both rabbit muscle and human erythrocyte phosphofructokinases (Karadsheh & Uyeda, 1977). Phosphorylation of phosphofructokinase, suggesting the existence of a specific protein kinase and phosphatase, was first reported by Brand & Soling (1975) from observations on liver extracts. Hofer & Furst (1976) isolated ^{32}P-labeled phosphofructokinase from the skeletal muscle of mice that had been injected with [^{32}P]P_i. However, phosphorylation of rabbit muscle phosphofructokinase, the enzyme source used in this study, apparently has no effect on catalytic activity (Hussey et al., 1977; Uyeda et al., 1978).

Several enzymes exhibit a reversible intracellular partitioning between soluble and particulate forms. Wilson (1978) has coined the work *ambiquitous* in reference to this phenomenon. The association of glycolytic enzymes with the structural proteins of muscle has been widely studied and reviewed (Clarke & Masters, 1976; Pette, 1978; Masters, 1978). Histochemical experiments have shown that the glycolytic and glycogenolytic enzymes are located within the I band of the muscle fiber, corresponding to the site of the thin filaments in the relaxed myofibril (Sigel & Pette, 1969; Arnold et al., 1969). Experiments with reconstituted thin filaments and with press juice extracts have demonstrated in vitro adsorption of several glycolytic enzymes, with phosphofructokinase being the most strongly adsorbed (Clarke & Masters, 1975). Actin is believed to be the major site of association (Arnold & Pette, 1968). Consistent with this is the observation that purified glycolytic enzymes such as aldolase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and others interact strongly with F-actin (Arnold et al., 1971). Significantly, the binding of aldolase to F-actin (Arnold & Pette, 1970) or to reconstituted thin filaments (Walsh et al., 1977) results in changes in its kinetic properties.

Accumulating evidence indicates that actin is very nearly a universal protein, comprising 10–20% of the cell protein in all eucaryotic cells (Korn, 1978). The association of glycolytic enzymes with actin may be a widespread phenomenon occurring in both muscle and nonmuscle cells. In view of this and of the key regulatory role of phosphofructokinase, we undertook activity measurements on the rabbit muscle enzyme in the presence of purified skeletal muscle F-actin and of reconstituted thin filaments. The results, showing major changes in the catalytic properties of phosphofructokinase, are the subject of this paper.

Materials and Methods

Reagents. ATP, ADP, AMP, fructose 6-phosphate and fructose 1,6-bisphosphate were obtained from Sigma Chemical Co.; glycylglycine and α-glycerophosphate were from Calbiochem. All other chemicals were reagent grade. All solutions were prepared by using deionized glass-distilled water.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was prepared by the method of Ling et al. (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate, pH 8.0, containing 1 mM EDTA and 1 mM dithiothreitol and dialyzed against the same buffer to give a stock enzyme solution of 10–15 mg/mL. The enzyme had a specific activity of 100–120 units/mg.

Before experiments the enzyme was dialyzed overnight against a buffer consisting of 25 mM glycylglycine, 25 mM sodium glycerophosphate, 1 mM EDTA, and 1 mM mercaptoethanol, pH 7.0. This buffer provides a degree of stability to the enzyme without interfering with the various kinetic parameters (Colombo et al., 1975). The dialysate was clarified by centrifugation.

Phosphofructokinase concentrations were determined by using $E_{283}^{1\%} = 10.9$ in 0.1 N NaOH (Paetkau et al., 1968). Molar concentrations of phosphofructokinase were based on a protomer molecular weight of 90000 (Colombo et al., 1975).

Actin. An acetone powder of rabbit back and leg muscle was prepared by the Straub procedures as modified by

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Szent-Gyorgyi (1947). The powder was dried at room temperature and stored in a tightly closed container at −10 °C.

Actin was purified according to the procedure of Spudich & Watt (1971). After a second polymerization, F-actin was centrifuged at 80000*g* for 3 h and the sediment was resuspended in a solution of 2 mM Tris-HCl, pH 8.0, containing 50 mM KCl. For resuspension, which was done in the cold, a small (7 mL) Dounce tissue grinder was used. The concentration of the resuspended F-actin was adjusted to ~10 mg of protein per mL. This preparation of F-actin was used within 24 h of the second polymerization.

The actin concentration was calculated by using an extinction coefficient value at 290 nm of 0.63 mg^{-1} mL cm^{-1} (Lehrer & Kerwar, 1972).

Tropomyosin. Bailey's (1948) method as modified by Mueller (1966) was followed by the preparation of tropomyosin B. The tropomyosin was collected between 40 and 55% ammonium sulfate saturation. It was then dissolved in 25 mM imidazole, pH 7.0, containing 60 mM KCl and 2 mM $MgCl_2$ and dialyzed overnight against the same solution. The concentration of tropomyosin was measured spectrophotometrically by using an extinction coefficient at 277 nm in 1.1 ionic strength buffer at pH 7.0 of 0.33 mg^{-1} mL cm^{-1} (Woods, 1967).

Troponin. Troponin was prepared according to the method of Greaser & Gergely (1973).

Reconstitution of the Thin Filament. The F-actin–tropomyosin–troponin complex was prepared according to the method of Ishiwata (1973) by mixing the three proteins in this order in the ratio 10:2:1 (by weight).

Activity Assay of Phosphofructokinase. The pH stat assay of Dyson & Noltmann (1965) was adopted for the determination of the catalytic activities of free and protein-bound phosphofructokinase. The standard reaction mixture contained final concentrations of 1 mM ATP, 1 mM F6P, 5 mM $MgCl_2$, and 10 mM KCl, adjusted to pH 7.00 in a total volume of 2.0 mL. After waiting a short period for the instrument to attain a steady pH reading, the reaction was initiated by the addition of a 10–25-μL aliquot of phosphofructokinase diluted to give ~0.1–0.2 unit/aliquot. The phosphofructokinase had been diluted with a buffer consisting of 12.5 mM glycylglycine, 12.5 mM glycerophosphate, and 0.5 mM EDTA, adjusted to pH 7.0, and preincubated at room temperature for 5 min to ensure maximal activity.

Base consumption as a function of time was controlled and recorded with a Type SBR2/SBu1/TTA3 titrator (Radiometer, Copenhagen). NaOH (2 mM) was used as titrant and was standardized against potassium hydrogen phthalate at the beginning of each experiment. The unit of phosphofructokinase activity is defined as that amount of enzyme which causes a reaction rate equal to the consumption of 1 μequiv of NaOH per min under the stated assay conditions.

Results

The experiments were designed to reveal possible effects of F-actin and the reconstituted thin filaments on some of the familiar regulatory properties of rabbit muscle phosphofructokinase. These include the sigmoid substrate saturation curve for fructose 6-phosphate, the inhibition found at high ATP concentrations, and the activating and inhibitory effects of AMP and citrate, respectively. We adapted the pH stat assay of Dyson & Noltmann (1965), in which the H^+ produced by the enzymatic reaction is titrated with standard NaOH.

$$(\text{fructose 6-phosphate})^{2-} + ATP^{4-} \rightarrow (\text{fructose 1,6-bisphosphate})^{4-} + ADP^{3-} + H^+$$

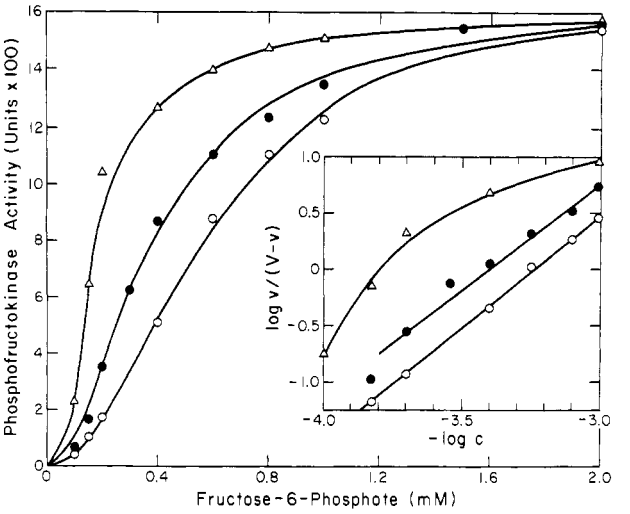

FIGURE 1: Effect of F-actin and the reconstituted thin filament on the substrate saturation curve for fructose 6-phosphate. Phosphofructokinase activity was assayed at pH 7 and 25 °C in the presence of 1 mM ATP, 10 mM KCl, and 5 mM $MgCl_2$. The enzyme concentration was in the range of 1 to 2 μg/mL. Additions made were none (○), 0.2 mg/mL reconstituted thin filament (●), and 0.2 mg/mL F-actin (△). The smooth curves (○ and ●) are theoretical curves calculated for the constants listed in Table I. Hill plots are shown in the inset.

Table I: Summary of Results When Fructose 6-Phosphate Is the Variable Substrate[a]

KCl (M)	additions (0.2 mg/mL)	$S_{1/2}$ (mM)	V_{max}[b] (units)	n
0.01	none	0.61	0.17	2.0
0.01	F-actin	0.16	0.16	>1[c]
0.01	reconstituted thin filament	0.40	0.16	1.84
0.14	none	0.16	0.14	1.9

[a] The solutions contained 1 mM ATP and 5 mM $MgCl_2$ at pH 7 and 25 °C. [b] V_{max} was obtained from extrapolation of linear plots of $1/v$ vs. $1/[\text{Fru-6-P}]^2$. [c] Hill plot was nonlinear. n was averaged in the range of 2.

This direct method avoids possible complications due to the auxiliary enzymes used in the standard coupled assays. Several of these enzymes (aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and lactic dehydrogenase) bind to F-actin (Arnold et al., 1971). The choice of experimental conditions, unbuffered 0.01 M KCl containing 5 mM $MgCl_2$ adjusted to pH 7.0, reflects the requirements of the assay and the ionic strength used in comparable studies of F-actin and thin filament bound aldolase (Arnold & Pette, 1970; Walsh et al., 1977).

The Fructose 6-Phosphate Saturation Curve. Figure 1 demonstrates the activating effects obtained on the addition of either F-actin or reconstituted thin filaments to rabbit muscle phosphofructokinase. The saturation curves are shifted to the left, with an apparent decrease in K_m for fructose 6-phosphate and little change in V_{max}. Cooperativity is maintained. Hill plots, shown in the inset, show considerable nonlinearity in the presence of F-actin. Table I summarizes the values of V_{max}, $S_{1/2}$, the substrate concentration of 50% V_{max}, and n, the Hill coefficient. The addition of bovine serum albumin to phosphofructokinase had no effect on enzymatic activity.

ATP Inhibition. Similar experiments using ATP as the variable substrate show that the presence of either F-actin or the reconstituted thin filament tends to reverse the inhibition observed at high ATP concentrations (Figure 2). The con-

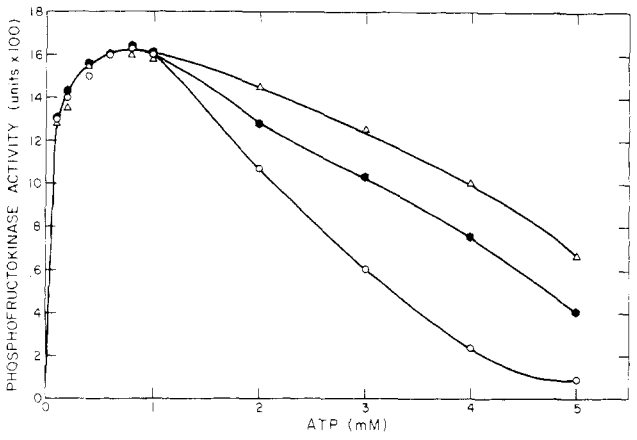

FIGURE 2: Effect of F-actin and the reconstituted thin filament on the ATP inhibition of phosphofructokinase. The fructose 6-phosphate concentration was fixed at 1 mM. Other conditions are listed in the caption to Figure 1. (○) No additions; (△) F-actin; (●) reconstituted thin filaments.

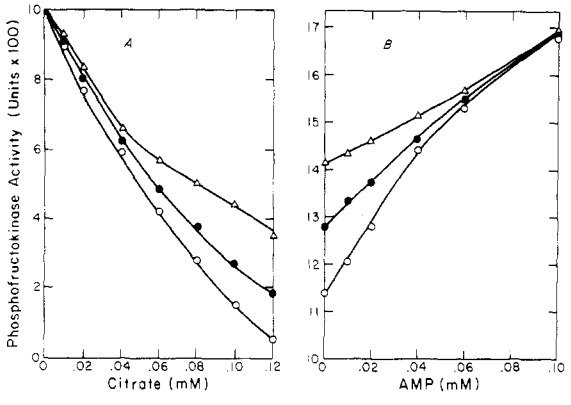

FIGURE 3: Reciprocity between the effects of F-actin or the reconstituted thin filament and allosteric effectors of phosphofructokinase. (A) Inhibition by citrate. The assay was carried out with 1 mM ATP and 1 mM fructose 6-phosphate. (B) Activation by AMP. The assay solution contained 2.5 mM ATP and 1 mM fructose 6-phosphate. Other conditions are described in the caption to Figure 1. (○) No additions; (△) F-actin; (●) reconstituted thin filaments.

centration of ATP required to produce 50% inhibition increases from 2.5 mM for free phosphofructokinase to 3.7 and 4.2 mM in the presence of the thin filaments and F-actin, respectively. The maximal activity of the enzyme at moderately high concentrations (1 mM) of both ATP and fructose 6-phosphate remains unaffected (±10%), however.

F-Actin and the thin filaments resemble other positive effectors of rabbit muscle phosphofructokinase in their ability to partially reverse ATP inhibition. We carried out experiments to determine whether there is any reciprocity between the effects shown in Figure 2 and those of AMP, a well-known activator, and of citrate, an inhibitor synergistic with ATP (Colombo et al., 1975). The effect of citrate was examined under conditions where the enzyme shows maximal activity (1 mM ATP). The concentration of citrate required to produce 50% inhibition under these conditions changes from 50 μM for free phosphofructokinase to 55 and 80 μM, respectively, when the reconstituted thin filament or F-actin is added (Figure 3A).

In the presence of an ATP concentration (2.5 mM) which reduces the free enzyme activity to one-half its optimal value, AMP activates the enzyme with maximal effect attained at 0.1 mM AMP (Figure 3B). This plateau in activity, corresponding to 75% of the activity obtained at optimal ATP concentrations, was shown to extend to 0.5 mM AMP. Figure

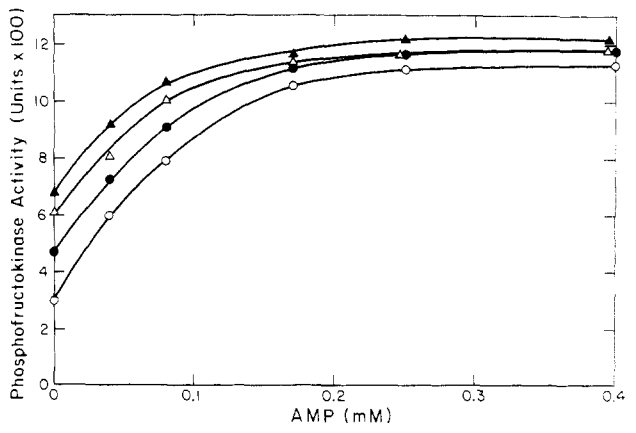

FIGURE 4: Sensitivity of phosphofructokinase to activation by AMP in the presence of varying concentrations of F-actin ranging from 0 (○), 0.05 (●), and 0.2 mg/mL (△) to (▲) 0.4 mg/mL. The assay solution contained 1 mM fructose 6-phosphate and 4 mM ATP. Other conditions are listed in the caption to Figure 1.

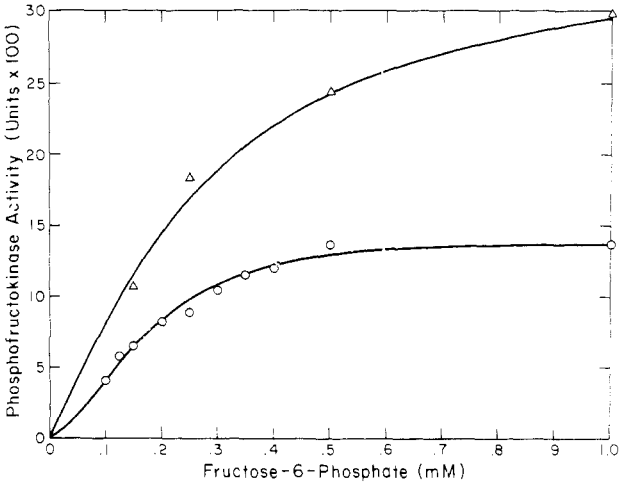

FIGURE 5: Effect of increased KCl concentration on the activation of phosphofructokinase by F-actin. The experiment shown in Figure 1 was repeated with 0.14 M KCl. (○) No additions; (△) 0.2 mg/mL F-actin.

3B shows that the maximum activation obtained using combinations of either F-actin or the thin filament with AMP is about the same as that obtained with AMP alone. This effect was examined more closely in experiments carried out at 4 mM ATP, which inhibits the free enzyme to less than 20% optimal activity. Varying concentrations of F-actin, up to 0.4 mg/mL, show progressive effects in reversing the ATP inhibition (Figure 4). However, the plateau in activity obtained at saturating AMP concentrations is nearly independent (within 10%) of the F-actin concentration. In none of these cases is the maximum activity of the enzyme restored.

Ionic Strength. The KCl concentration was increased to 0.14 M in order to see whether the activation of phosphofructokinase by F-actin persists at physiological ionic strength. Figure 5 shows that KCl itself has an activating effect, with a decrease in the K_m for fructose 6-phosphate, similar to that observed by Otto et al. (1976) under different conditions. Nonetheless, the addition of F-actin causes a further enhancement of activity with possible alterations in K_m and V_{max} as well as in the Hill coefficient.

Discussion

The present study shows that F-actin and the reconstituted thin filament exert striking effects on the catalytic properties

of rabbit muscle phosphofructokinase. The addition of either partially reverses the inhibition of phosphofructokinase seen at high ATP concentrations and increases the apparent affinity of the enzyme for fructose 6-phosphate with slight change in V_{max}. In this respect, F-actin and the thin filament resemble positive effectors of phosphofructokinase such as AMP. The *maximum* effect in the reversal of ATP inhibition is about the same for combinations of either F-actin or the thin filament with AMP as it is for AMP alone. This observation contrasts with the marked synergism seen in the joint activation of rat muscle phosphofructokinase by AMP and fructose 1,6-bisphosphate (Tornheim & Lowenstein, 1976). Possibly F-actin and AMP activate phosphofructokinase through a common mechanism. However, details of the activation obtained with F-actin differ from those obtained with AMP. The cooperativity with respect to fructose 6-phosphate is maintained after activation by F-actin but lost after activation by AMP (Odeide et al., 1969). The persistence of cooperativity is reminiscent of the observations of Hofer (1970), who found that the sensitivity to ATP inhibition and the K_m for fructose 6-phosphate both decrease with increasing phosphofructokinase concentration while cooperativity is maintained. Hofer concluded that, under the conditions of his experiments, polymers of the enzyme larger than the tetramer have varying allosteric properties.

F-Actin is more effective than the reconstituted thin filament, which is 77% actin by weight, in activating phosphofructokinase. With aldolase, the effect of the thin filament is larger than that of F-actin (Arnold & Pette, 1970; Walsh et al., 1977). The activation of phosphofructokinase by F-actin and the thin filament resembles that obtained by Karadsheh & Uyeda (1977) with the red blood cell membrane. As with AMP, the major difference between the two cases pertains to the cooperativity. Although actin is a major component of the red blood cell membrane (Marchesi et al., 1976), Richards et al. (1979) have evidence that the activation obtained with the membrane is due instead to the band 3 protein.

Low concentrations of fructose 1,6-bisphosphate and ADP, allosteric activators of phosphofructokinase, are inevitably present in these assays. The conversion of G- to F-actin is accompanied by the hydrolysis of ATP, leaving ADP tightly bound to F-actin. At the F-actin concentrations used in these experiments (4×10^{-6} M in terms of the G-actin monomer), the concentration of unbound ADP is likely to be far below that required to activate the enzyme. Since bound ADP is an integral part of F-actin, it is difficult to distinguish its effects from those of the protein moiety. The linearity of the time courses indicated that the changing concentrations of ADP and fructose 1,6-bisphosphate did not influence the relative rates obtained in the presence and absence of F-actin. The detection of possible synergism in the effects of F-actin and fructose 1,6-bisphosphate requires a more sensitive assay method giving "zero-time" rates.

The stoichiometry of binding of phosphofructokinase to F-actin is the object of measurements now being carried out in our laboratory. Preliminary results indicate that the binding ratio in vitro can exceed 1 phosphofructokinase tetramer/actin monomer (Liou, 1979). Liou suggested that phosphofructokinase forms linear polymers attached at one or both ends to F-actin. However, a high degree of saturation of F-actin with bound phosphofructokinase is unlikely to occur either in muscle or under the conditions of our activity measurements. The physiological concentrations of F-actin [25–30 mg/g of muscle (Hasselbach & Schneider, 1951)] and phosphofructokinase [0.33 mg/g (de Duve, 1972)] correspond to ~700 actin monomers/phosphofructokinase tetramer. A similar ratio pertains to our activity measurements, where the F-actin concentration was usually 0.2 mg/mL and the phosphofructokinase concentration, 1–10 μg/mL.

The adsorption of glycolytic enzymes to particulate fractions generally has been studied at low ionic strength. The physiological significance of the results has not been unanimously accepted since increases in ionic strength diminish the adsorption detected in vitro. However, Clarke & Masters (1975) point out that these experiments also use reduced protein concentrations and that adsorption is enhanced at higher concentrations. An experiment which we conducted at near-physiological ionic strength shows that the activating effect of F-actin on phosphofructokinase persists. Although the results differ in detail, the activation obtained at both low and high ionic strength, with fructose 6-phosphate as the variable substrate, spans the range of physiological fructose 6-phosphate concentrations. Data tabulated by Arnold & Pette (1970) indicate that the levels of fructose 6-phosphate range from ~0.29 mM in resting muscle to ~1.3 mM in contracting muscle.

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Kinetics of Oxygen and Carbon Monoxide Binding to the Hemoglobins of *Glycera dibranchiata*†

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ABSTRACT: The monomeric hemoglobin fraction from *Glycera dibranchiata*, the blood worm, has been fractionated into four components. Rate constants and activation energies have been measured for O_2 and CO association and dissociation reactions for the three most abundant monomeric hemoglobins. Hemoglobin I appears to have the largest O_2 and CO association and O_2 dissociation rate constants yet reported for a hemoglobin. Hemoglobin II shows very similar kinetics. The values for M, the O_2–CO partition constant, for these two components appear to be the largest for any hemoglobin. The CO and O_2 association and O_2 dissociation rate constants for hemoglobin III, the most basic of the three major monomeric hemoglobins, are about one-sixth to one-eighth those for hemoglobins I and II, a striking example of functional heterogeneity. On the other hand, oxygen equilibrium constants are very similar for the three hemoglobins. It appears that hemoglobin II corresponds to that hemoglobin for which the sequence has been reported. The unusually rapid ligand kinetics are thus associated with the substitution of the distal histidine by leucine. In all cases, the monomer ligand kinetics were monophasic, in contrast to earlier results [Seamonds, B., McCray, J. A., Parkhurst, L. J., & Smith, P. D. (1976) *J. Biol. Chem. 251*, 2579]. The O_2 and CO association ligand kinetics for the polymer were at least biphasic. The rates for the faster component were similar to those for hemoglobin III of the monomer and about four times faster than the rates for the slower polymeric component. CO dissociation was monophasic for the polymer. Analysis of the O_2–CO relaxation data suggests that there is very little heterogeneity in the O_2 dissociation reaction for the polymeric component.

Seamonds (1969) and Seamonds et al. (1971a) have reported the occurrence of monomeric and polymeric hemoglobins in the erythrocytes of the bloodworm *Glycera dibranchiata*. Imamura et al. (1972) have reported the sequence of one of the monomeric hemoglobins, and Padlan & Love (1974) have determined the crystal structure of the same hemoglobin to 2.5-Å resolution. They noted that 79% of the 147 residues occur in helical regions and that the D helix is absent, as in the α chains of vertebrate hemoglobins. A feature of interest is the replacement of the distal histidine by leucine in the monomer sequence (Imamura et al., 1972). Seamonds et al. (1976) reported oxygen association (k') and both CO association (l') and dissociation (l) rate constants for the total monomeric fraction and noted that the association kinetics were at least biphasic. Since the biphasicity was not consistent with earlier electrophoretic results, the authors were led to propose equilibria among monomeric conformers, a suggestion supported by electron paramagnetic resonance (EPR) measurements (Seamonds et al., 1972). This lack of agreement between kinetic and electrophoretic results also prevented analysis of the O_2–CO replacement data so as to obtain dissociation rate constants (k) for oxygen, since the mechanism for the ligand binding reactions was unclear. In this paper, we report the isolation of four components from the monomeric fraction of *G. dibranchiata* hemoglobin and report oxygen and CO association and dissociation rate constants and activation energies for the three principal components. The same four

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