2740-2743.
Studier, F. W. (1965), *J. Mol. Biol. 11,* 373-390.
Varshavsky, A. J., Bakayev, V. V., Ilyn, Y. V., Bayer, A. A., and Georgiev, G. P. (1976), *Eur. J. Biochem. 66,* 211-223.
Varshavsky, A. J., Ilyin, Y. V., and Georgiev, G. P. (1974), *Nature (London) 250,* 602-606.
Vesterberg, O. (1971), *Methods Enzymol. 22,* 389-412.
Weinman, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790-1794.

Properties of Rabbit Muscle Phosphofructokinase Modified with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole†

P. M. Lad, Neil M. Wolfman,‡ and Gordon G. Hammes*

ABSTRACT: A single sulfhydryl group per polypeptide chain of rabbit muscle phosphofructokinase has been specifically labeled with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) at pH 7. The rate of reaction is similar in the presence of fructose 6-phosphate, fructose 1,6-bisphosphate, Mg^{2+}, and low ATP concentrations (100 μM) but is markedly decreased by high concentrations of MgATP (5 mM). At higher molar ratios of reagent to protomer (5:1 vs. 2.5:1) more than a single sulfhydryl group is labeled. The labeled enzyme has a specific activity 15-30% that of the native enzyme. Removal of NBD from the enzyme by dithiothreitol only partially restores the activity (up to 60%) although the circular dichroism and aggregation state of the protein are unchanged by the chemical modification. The modified enzyme has a considerably larger Michaelis constant for MgATP (six- to tenfold) than the native enzyme at both pH 7.0 and 8.0. The inhibition constant for adenylyl imidodiphosphate also increases. The modified enzyme is strongly inhibited by MgATP at pH 7.0 but at concentrations approximately twice as large as with the native enzyme. The Michaelis constant for fructose 6-phosphate is approximately the same for both the native and modified enzyme at pH 8.0. However, at pH 7.0 the cooperativity associated with fructose 6-phosphate binding is considerably reduced with the Hill coefficient changing from 4.0 to 1.4. The location of the modified sulfhydryl group with respect to the MgATP catalytic site and inhibitory site is uncertain since modification has a major effect on both sites.

Rabbit muscle phosphofructokinase is a key enzyme in the regulation of glycolysis. The enzyme activity is altered by a number of metabolites (Passoneau and Lowry, 1962, 1963) and the steady kinetic properties of the enzyme are complex at pH 7 and below (Hofer and Pette, 1968), suggesting the enzyme has allosteric properties. The enzyme is made up of apparently identical subunits of molecular weight 80 000 (Leonard and Walker, 1972; Pavelich and Hammes, 1973; Coffee et al., 1973), and its regulation apparently involves both aggregation phenomena and conformational changes within a tetramer or higher aggregate (Lad et al., 1973; Hill and Hammes, 1975; Frieden et al., 1976).

In the work reported here, a single sulfhydryl group (per subunit of mol wt 80 000) has been modified with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, NBD-Cl,[1] and the effects of this modification on the kinetic properties of the enzyme have been determined. The results obtained indicate that the primary effects of modifying the sulfhydryl group are to decrease the affinity of the enzyme for MgATP (also for AMP-PNP and MgAMP-PNP) at the catalytic site, to reduce the cooperativity in the fructose 6-phosphate-enzyme interaction at pH 7.0, and to reduce the catalytic activity to 15-30% of that of the native enzyme.

Experimental Section

Chemicals. The ATP, ADP, fructose 6-phosphate, fructose 1,6-bisphosphate, dithiothreitol, aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, and bovine serum albumin were purchased from Sigma Chemicals. The AMP-PMP was obtained from P-L Biochemicals. The NBD-Cl was obtained from Pierce Chemical Co. All other chemicals were the best available commercial products, and all solutions were prepared with deionized distilled water.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling et al. (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate (pH 8.0), 1.0 mM EDTA and dialyzed against the same buffer to give a stock solution of 10-14 mg/mL. The enzyme concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.02 mL mg^{-1} cm^{-1} (Parmeggiani et al., 1966). The specific activity of the enzyme at 23 °C (33 mM Tris-Cl, 2 mM fructose 6-phosphate, 2 mM ATP, 5 mM $MgCl_2$, pH 8.0) was 100-120 units/mg. (A unit of enzyme activity is defined as the formation of 1 μmol of product/min.) After a period of about 4 weeks, the specific activity of the enzyme stock solution declines significantly. Only enzyme of specific activity greater than 90 units/mg was employed to obtain the results presented here.

Reaction of Phosphofructokinase with NBD-Cl. The reaction of the enzyme with NBD-Cl was carried out in 25 mM diglycine, 25 mM potassium phosphate (pH 7.2), 0.4 mM fructose 6-phosphate, 0.1 mM ATP, 1 mM EDTA at 23 °C or in the same buffer at pH 7.0 with 5 mM fructose 6-phos-

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received May 10, 1977.* This work was supported by a grant from the National Institutes of Health (GM 13292).
‡ National Institutes of Health Predoctoral Trainee (GM 00834).
[1] Abbreviations used are: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AMP-PNP, adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid.

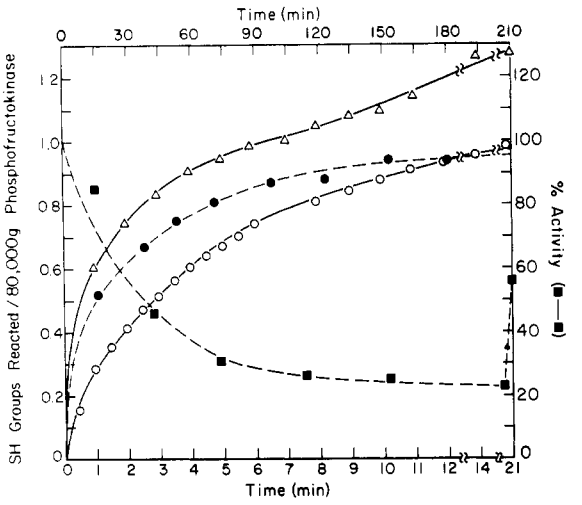

FIGURE 1: The number of sulfhydryl groups reacted vs. time after addition of NBD-Cl to phosphofructokinase. The enzyme was incubated at a concentration of 0.95 mg/mL in 25 mM diglycine, 25 mM potassium phosphate (pH 7.2), 0.4 mM fructose 6-phosphate, 0.1 mM ATP, 1 mM EDTA at 23 °C (open symbols, lower abscissa) or in 25 mM diglycine, 25 mM potassium phosphate (pH 7.0), 5 mM fructose 6-phosphate, 5 mM ATP, 1 mM EDTA at 25 °C (●, upper abscissa). The NBD-Cl (20 mM in dimethyl sulfoxide) was added to a final molar ratio of 2.5 (○, ●) or 5 (△) relative to the enzyme protomer (mol wt 80 000). The decrease in activity paralleling the modification also is shown (■ upper abscissa, right ordinate). The point at 210 min indicates the increase in activity after addition of dithiothreitol to 0.5 mM. The standard assay conditions are given in the Experimental Section.

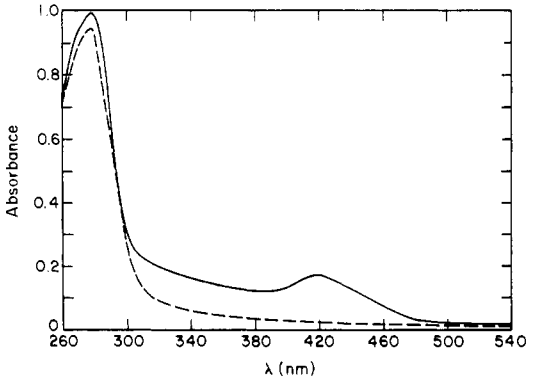

FIGURE 2: The absorption spectra of the NBD-Cl modified (—) and native (- - -) enzymes. The enzyme concentration is 0.95 mg/mL in 0.1 M phosphate (pH 8.0), 1 mM EDTA. The modified and native enzyme were dialyzed overnight against the buffer. The molar ratio of NBD-Cl bound to the enzyme protomer is 0.90.

phate, 5 mM ATP, 1 mM EDTA at 25 °C. The NBD-Cl was dissolved in dimethyl sulfoxide or absolute ethanol to give a 10–40 mM solution. The reaction was initiated by adding a small aliquot of this NBD-Cl solution to the enzyme solution. The extent of reaction was monitored spectrophotometrically at 420 nm. The reaction was stopped by dialysis of the reaction mixture against 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA. The moles of NBD-Cl reacted were determined assuming an extinction coefficient of 13 000 M^{-1} cm^{-1} at 420 nm for the NBD-Cl bound to the enzyme (Birkett et al., 1971). In some cases the NBD-Cl modified enzyme was concentrated either by precipitation with 60% saturated ammonium sulfate or ultrafiltration under nitrogen in a Diaflow cell. The specific activity of the modified enzyme was not appreciably altered by either procedure.

Steady-State Kinetics. The phosphofructokinase was assayed spectrophotometrically as previously described (Ling et al., 1966; Lad et al., 1973). The steady-state kinetic studies of the native and NBD-Cl modified enzymes were carried out at 23 °C, pH 7.0 and 8.0 in 33 mM Tris-Cl, 0.1 mM NADH, 1 mM dithiothreitol (this was excluded in studies with the modified enzyme), 0.25 unit/mL aldolase, 3.2 units/mL of α-glycerophosphate dehydrogenase, 35 units/mL triose phosphate isomerase, and 0.1–0.3 μg/mL phosphofructokinase with varying amounts of $MgCl_2$, ATP, fructose 6-phosphate, and AMP-PNP. In addition 67 μM potassium phosphate was present; this is the amount of potassium phosphate introduced from the enzyme stock solution. All assays were initiated by the addition of phosphofructokinase to the reaction mixture, and the velocity of the enzymatic reaction was recorded spectrophotometrically at 340 nm on a Cary 14 spectrophotometer.

Spectral Measurements. The ultraviolet absorption spectra of the native and modified enzymes were determined on a Zeiss PMQII spectrophotometer. The circular dichroism spectra were determined at 23 °C on a Cary 60 spectropolarimeter using cells of 3-cm path length.

Gel Chromatography. The Stokes' radii of the native and modified enzymes were determined by column chromatography on Agarose (1.5m, Bio-Rad Lab; 1.5 cm i.d. × 50 cm) using previously described methods (Pavelich and Hammes, 1973).

Results

Reaction of NBD-Cl with Phosphofructokinase. The addition of NBD-Cl (2.5 molar ratio to enzyme) to the enzyme causes an increase in the absorbance at 420 nm, and the number of sulfhydryl groups reacted as a function of time is shown in Figure 1. One sulfhydryl group per subunit (mol wt 80 000) is much more reactive than other groups. As is apparent from the results shown, the reaction is slower and more specific for a single sulfhydryl group at higher ATP and fructose 6-phosphate concentrations and lower pH. The decrease in enzyme activity during the course of the reaction also is presented in Figure 1. Typically 15–30% of the initial activity remains when one sulfhydryl group has been modified. A kinetic analysis of the time dependence of the absorbance changes indicates the reaction is first order with respect to both NBD-Cl and enzyme. The second-order rate constant in 25 mM diglycine, 25 mM potassium phosphate (pH 7.2), 0.4 mM fructose 6-phosphate, 1 mM ATP, 1 mM EDTA at 23 °C is 149 M^{-1} s^{-1}; in 25 mM diglycine, 25 mM potassium phosphate (pH 7.0), 5 mM ATP, 5 mM fructose 6-phosphate, 1 mM EDTA, it is 18 M^{-1} s^{-1}. The absorption spectrum of the enzyme after overnight dialysis with about 1 mol of NBD-Cl reacted per subunit is shown in Figure 2. The characteristic absorption peak at 420 nm confirms that NBD-Cl has reacted with a sulfhydryl group (Birkett et al., 1971). If a large excess of NBD-Cl is used, a slower reaction with other sulfhydryl groups also occurs (Figure 1). The modified enzyme employed in the kinetic measurements reported later had 0.85–1.0 mol of NBD-Cl bound per subunit (mol wt 80 000).

The reactivity of the first sulfhydryl group is influenced by the presence of substrates and regulatory effectors. The value of the rate constant in the presence of various ligands is presented in Table I. The enzyme, present at a concentration of 0.7–1 mg/mL, is primarily a tetramer in all cases (Lad et al., 1973).

Kinetic Studies. The steady-state kinetic properties of the native and modified enzymes at pH 7.0 and 8.0, 23 °C, are summarized in Table II. The apparent Michaelis constants for

TABLE I: Rate Constants for the Reaction of NBD-Cl with Phosphofructokinase.[a]

Substrate or effector	Rate constant (M^{-1} s^{-1})
KP_i, 25 mM; Fru 6-P, 0.4 mM; ATP, 0.1 mM	149
Fru 6-P, 5 mM	83.3
$MgCl_2$, 5 mM	163
Fru 1, 6-P_2, 5 mM	83.3
KP_i, 20 mM	102
AMP, 5 mM	78.3
MgATP, 5 mM	7.5
$MgCl_2$, 5 mM; AMP-PNP, 250 μM	44.5
$MgCl_2$, 5 mM; ATP, 100 μM	104
AMP-PNP, 250 μM	93.7
ATP, 100 μM	98.8

[a] With 25 mM diglycine (pH 7.2), 1 mM EDTA, 1 mg/mL phosphofructokinase, 3.12×10^{-5} M NBD-Cl, 23 °C.

TABLE II: Kinetic Parameters of Native and NBD-Cl Modified Phosphofructokinase.[a]

Parameter	pH	Native	Modified
K_{mMgATP} (mM)	8.0[b]	0.019	0.183
	7.0[c]	0.028	0.158
K_{mF6P} (mM)	8.0[d]	0.097	0.136
$K_{1/2F6P}$ (mM)	7.0[e]	3.7	0.55
V_{max} (units/mg)[f]	8.0[d]	95	15
	7.0[e]	33	6
K_{iMgATP}	7.0[g]	0.41	0.88
$K_{iAMP-PNP}$	8.0[b]	0.022	0.345
	7.0[c]	0.0057	0.099

[a] 33 mM Tris-Cl, 1 mM dithiothreitol, 67 μM potassium phosphate, 23 °C. [b] 1 mM fructose 6-phosphate, 5 mM $MgCl_2$; 6 to 50 μM ATP, 0 to 122 μM AMP-PNP for the native enzyme; 0.05 to 0.30 mM ATP and 0 to 8 mM AMP-PNP for the modified enzyme. [c] 1 mM fructose 6-phosphate, 5 mM $MgCl_2$, 13.3 to 100 μM ATP, 0 to 20 mM AMP-PNP for the native enzyme; 15 μM to 100 μM ATP and 0 to 80 μM AMP-PNP for the modified enzyme. [d] 2 mM ATP, 5 mM $MgCl_2$, 0.006 to 0.33 mM fructose 6-phosphate for the native enzyme and 0.1 to 1.00 mM fructose 6-phosphate for the modified enzyme. [e] 2.7 mM MgATP for native enzyme and 4.0 mM MgATP for modified enzyme, 0.06 to 4.00 mM fructose 6-phosphate for native and modified enzymes. [f] Average of five preparations. [g] 1 mM fructose 6-phosphate, 0.10 to 1.33 mM MgATP for the native and 0 to 4 mM for the modified enzymes.

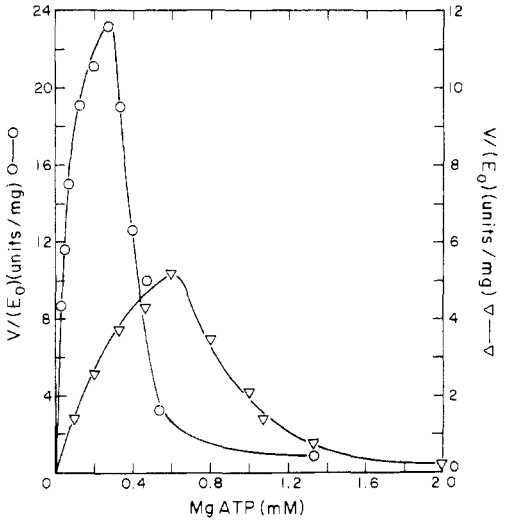

FIGURE 3: A plot of the steady-state initial velocity divided by the total concentration, $v/(E_0)$, of the native (○) and NBD-Cl modified (▽) enzymes vs. the MgATP concentration. The initial velocities were measured in 33 mM Tris-Cl (pH 7.0), 1 mM dithiothreitol (present only for the native enzyme), 1 mM fructose 6-phosphate at 23 °C. (Details are given in the Experimental Section.)

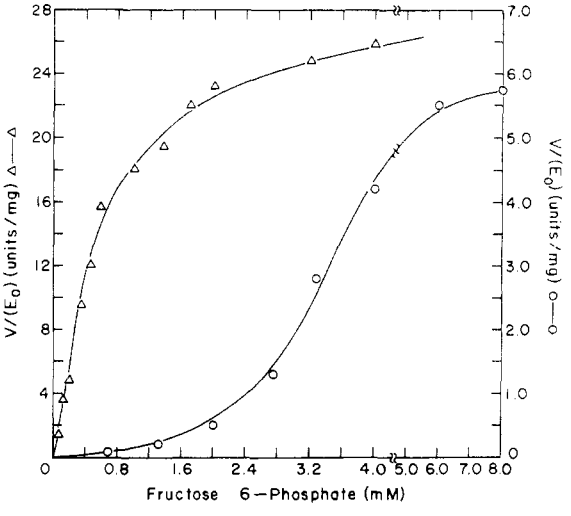

FIGURE 4: A plot of the steady-state initial velocity divided by the total enzyme concentration, $v/(E_0)$, for the native (○) and the NBD-Cl (▽) modified enzymes vs. the fructose 6-phosphate concentration. The initial velocities were measured in 33 mM Tris-Cl (pH 7.0), 2.7 mM MgATP for the native enzyme and 4.0 mM MgATP for the NBD-Cl modified enzyme at 23 °C. For the native enzyme 1 mM dithiothreitol also was present. (Details are given in the Experimental Section.)

fructose 6-phosphate (at constant MgATP concentration), K_{mF6P}, and for MgATP (at constant fructose 6-phosphate concentration), K_{mMgATP}, and the maximum velocities, V_m, were determined from plots of the reciprocal initial velocity vs. the reciprocal substrate concentration using a weighted least-squares analysis under conditions where simple Michaelis–Menten kinetics were observed, namely, at pH 8.0 and with MgATP as the varied substrate (at sufficiently low MgATP concentrations) at pH 7.0. The maximum velocity of the modified enzyme is drastically reduced relative to that of the native enzyme. However, the dependence of the initial reaction velocity on the MgATP concentration (1 mM fructose 6-phosphate) is qualitatively similar for both native and modified enzymes (Figure 3), except that the apparent Michaelis constant for MgATP is increased in the modified enzyme and the MgATP inhibition at pH 7.0 occurs at higher concentrations for the modified enzyme. In Table II, the MgATP inhibition at pH 7.0 is characterized by the MgATP concentration at which the initial velocity is reduced to one-half its maximal value (in the presence of 1 mM fructose 6-phosphate); this concentration is defined as K_{iMgATP}. At pH 8.0, the dependence of the initial velocity on the fructose 6-phosphate concentration obeys simple Michaelis–Menten kinetics. However, at pH 7.0, this is not the case as shown in Figure 4. The kinetic isotherms in these cases are characterized in Table II by the concentration of fructose 6-phosphate at which the initial velocity is one-half its maximal value, $K_{1/2}$. The kinetic isotherm of the native enzyme is characterized by a Hill coefficient of 4.0, while that for the modified enzyme is 1.4, which indicates the apparent cooperativity for fructose 6-phosphate interacting with the enzyme is greatly reduced in the modified enzyme. The ATP analogue AMP-PNP was found to be a competitive inhibitor of MgATP at both pH 7.0 and 8.0 for both the native and modified enzyme, and the competitive inhibition constants, $K_{iAMP-PNP}$, obtained from a least-squares analysis of the data are included in Table II.

The NBD can be readily removed from the enzyme by the addition of dithiothreitol to the enzyme solution. If this is done in the reaction mixture after one sulfhydryl group has been modified, activity is only partially restored. A typical result is shown in Figure 1. The maximum restoration of activity is to about 60% of the original activity. This phenomenon was examined in more detail by dialyzing the modified enzyme overnight against 0.1 M potassium phosphate (pH 7.0), 1 mM EDTA at 5 °C. The enzyme concentration was adjusted to 0.15 mg/mL and a concentrated aliquot of dithiothreitol was added to the solution to a final concentration of 0.5 mM or greater. The activity of the enzyme slowly increased over a 2-h period but never reached higher than about 60% of the initial activity. The slowness of the regain of activity is not due to slow removal of the NBD as the absorbance at 420 nm disappears essentially instantaneously after the dithiothreitol is added.

Circular Dichroism and Stokes' Radii Measurements. The possibility exists that modification of a single sulfhydryl group per subunit causes gross structural changes. However, the ultraviolet circular dichroism spectra of the native and modified enzymes are essentially identical, displaying minima at 210, 220 and 270 nm (Leonard and Walker, 1972). This indicates large structural alterations have not occurred. The Stokes' radius at pH 8.0 in 0.1 M potassium phosphate as measured by gel filtration on agarose is the same as the native enzyme at identical protein concentrations (0.15 mg/mL), indicating no marked changes in the aggregation properties of the enzyme have occurred due to modification of the sulfhydryl group. Finally although the modified enzyme has a considerably reduced catalytic activity, the pH dependence of the initial velocity of the native and NBD-Cl modified enzyme in 33 mM Tris-Cl, 2 mM fructose 6-phosphate, 2 mM ATP, and 2 mM $MgCl_2$ are quite similar over the pH range 7.0 to 8.4.

Discussion

Although NBD-Cl can react with amino, tyrosine, and sulfhydryl groups, the spectrum of the product clearly identifies the reactive protein group as a sulfhydryl group, and the time course of the reaction indicates a product with a single modified sulfhydryl group can be readily isolated. The rate of the reaction of enzyme with NBD-Cl is strongly influenced by the presence of substrates and effectors (Table I). In general, the rate constant is similar in the presence of activators of the enzyme (Mg^{2+}, fructose 6-phosphate, fructose 1,6-bisphosphate, phosphate, AMP) but MgATP and MgAMP-PNP at high concentrations significantly decrease the rate constant. However, MgATP at low concentrations and ATP and AMP-PNP in the absence of Mg^{2+} have very little effect on the rate constant. In all cases a single sulfhydryl group can be selectively modified, and the reaction rate is second order. Although MgATP alters the rate of the reaction, it does not prevent the reaction from occurring stoichiometrically. The dependence of the rate constant on the concentration of MgATP and the requirement of both Mg and ATP or AMP-PNP for a significant decrease in the rate constant suggests that binding at the allosteric MgATP inhibitory site significantly reduces the reactivity of the sulfhydryl group, while binding at the catalytic site has little influence on the reactivity. The metal ion Mg^{2+} alone influences the rate constant suggesting the Mg^{2+} interacts directly with the enzyme, as indicated previously by kinetic (Hofer and Pette, 1968) and Stokes' radii measurements (Lad et al., 1973). The pattern of reactivity observed with NBD-Cl is similar to that found with other sulfhydryl group reagents (Kemp, 1969a,b; Schwartz et al., 1976).

The steady-state kinetic parameters summarized in Table II indicate that the NBD-Cl modified enzyme has a much lower activity than the native enzyme at both pH 7.0 and 8.0. The strength of the interaction between the enzyme and fructose 6-phosphate does not appear to be greatly altered in the modified enzyme as judged by the Michaelis constants at pH 8.0, although the cooperativity in the kinetic isotherm at pH 7.0 is greatly reduced. However, the Michaelis constant for MgATP is five to ten times larger for the modified enzyme, while the MgATP inhibition "constant" is increased about a factor of two. The competitive inhibition constant for AMP-PNP increases more than a factor of ten for the modified enzyme. Thus the major effect of modifying the sulfhydryl group with NBD-Cl on the substrate binding sites is to decrease the affinity of the catalytic site for MgATP and to reduce the cooperativity in the interaction of the enzyme with fructose 6-phosphate at pH 7.0. The affinity of the inhibitory site for MgATP also is decreased by the sulfhydryl group modification, but to a lesser extent.

Although the catalytic activity of the modified enzyme is greatly reduced, the similarity of the circular dichroism spectra, the initial velocity–pH dependencies and the Stokes' radii of the native and NBD-Cl modified enzymes indicate that gross structural changes in protein structure have not occurred. The inability to completely restore the enzyme activity when NBD is removed by dithiothreitol cannot be interpreted unequivocally; it may be that only a portion of the enzyme has full activity restored, or it may be that all of the enzyme is only partially reactivated. Why the reactivation should be slow and incomplete is unknown. The binding of MnATP to the enzyme has been reported to be weakened when the sulfhydryl group is modified with a spin label (4-(2-iodoacetamido)-2,2,6,6-tetramethyl piperidinooxyl; Jones et al., 1973). However, evidence indicating that the catalytic site is primarily affected has not previously been presented. The actual location of the NBD-Cl modified sulfhydryl group relative to the catalytic and inhibitory sites is unknown: it could be adjacent to either site or the alteration in the sulfhydryl group reactivity and the binding affinity could be due to conformational changes with the modified sulfhydryl being quite far from one or both of the sites.

References

Birkett, D. J., Dwek, R. A., Radda, G. K., Richards, R. E., and Salmon, A. G. (1971), *Eur. J. Biochem. 20,* 494.

Coffee, C. J., Aaronson, R. P., and Frieden, C. (1973), *J. Biol. Chem. 248,* 1381.

Frieden, C., Gilbert, H. R., and Bock, P. E. (1976), *J. Biol. Chem. 251,* 5644.

Hill, D. E., and Hammes, G. G. (1975), *Biochemistry 14,* 203.

Hofer, H. W., and Pette, D. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349,* 1378.

Jones, R., Dwek, R. A., and Walker, I. O. (1973), *Eur. J. Biochem. 34,* 28.

Kemp, R. G. (1969a), *Biochemistry 8,* 3162.

Kemp, R. G. (1969b), *Biochemistry 8,* 4490.

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

Leonard, K. R., and Walker, I. O. (1972), *Eur. J. Biochem. 26,* 442.

Ling, K. H., Paetkau, V., Marcus, F., and Lardy, H. A. (1966), *Methods Enzymol. 9,* 425.

Parmeggiani, A., Luft, J. M., Love, D. S., and Krebs, E. G. (1966), *J. Biol. Chem. 241,* 4625.

Passoneau, J. V., and Lowry, O. H. (1962), *Biochem. Biophys. Res. Commun. 7*, 10.
Passoneau, J. V., and Lowry, O. H. (1963), *Biochem. Biophys. Res. Commun. 13*, 372.
Pavelich, M. J., and Hammes, G. G. (1973), *Biochemistry 12*, 1408.
Schwartz, K. L., Nakagawa, Y., and Kaiser, E. T. (1976), *J. Am. Chem. Soc. 98*, 6369.

Fluorescence Energy Transfer Measurements in Rabbit Muscle Phosphofructokinase†

Neil M. Wolfman‡ and Gordon G. Hammes*

ABSTRACT: The distance between the citrate site and the most reactive sulfhydryl group in rabbit muscle phosphofructokinase has been determined using fluorescence resonance energy transfer. Pyridoxal 5′-phosphate and sodium borohydride reduction were used to specifically label a lysine residue at the citrate site, while 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) or 4-dimethylamino-4′-maleimidostilbene (NSM) was reacted with the most reactive sulfhydryl group of the monomeric enzyme (molecular weight 80 000). Using pyridoxamine 5′-phosphate as the fluorescent donor molecule, the measured distance was 40 Å with NBD as the acceptor molecule and 36 Å with NSM as the acceptor molecule. The magnitude of the transfer efficiency to NBD was unaltered by the presence of varying ATP concentrations (0.01–1 mM), 5 mM fructose 6-phosphate and 5 mM $MgCl_2$, and was unaffected by the aggregation state of the enzyme. Pyridoxylation of the enzyme led to depolymerization to dimer (pH 8.0), and to dimer and monomer (pH 7.0) at a protein concentration of 0.2 mg/mL, but incubation of the modified enzyme with 5 mM fructose 6-phosphate at pH 7.0 resulted in formation of the tetramer. Since the energy transfer efficiency is not dependent on the aggregation state of the enzyme, the predominant energy transfer probably occurs within a single polypeptide chain. If energy transfer occurs between subunits, the calculated distances are lower bounds to the distance within a single subunit.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is an important regulatory enzyme. The activity of the enzyme is dependent upon the pH, the protein concentration, and the presence or absence of several effectors (Passoneau and Lowry, 1962; Aaronson and Frieden, 1972; Pavelich and Hammes, 1973; Lad et al., 1973). Phosphofructokinase is a multisubunit protein containing identical polypeptide chains with a monomer molecular weight of 80 000 (Pavelich and Hammes, 1973; Coffee et al., 1973), and the specific activity of the enzyme is dependent on its aggregation state. Several models have been proposed to explain the regulatory properties of the enzyme (Kemp et al., 1976; Bock and Frieden, 1976).

In this work, fluorescence energy transfer measurements have been used to determine the distance from the citrate binding site to the most reactive sulfhydryl group (Kemp and Forest, 1968) in the presence of various effectors. Pyridoxamine 5′-phosphate (PMP),[1] which has been shown to bind specifically to a lysine residue at the citrate site (Colombo and Kemp, 1976), was used as the fluorescent donor while NBD-Cl and NSM reacted with the thiol group were used as the energy acceptrs. The distance between the two sites was found to be about 40 Å for the enzyme over a range of aggregation states, in the presence of varying ATP concentrations, in the presence of fructose 6-phosphate, and in the presence of $MgCl_2$.

Experimental Section

Materials. The ATP, fructose 6-phosphate, dithiothreitol, aldolase, glucose oxidase, α-glycerophosphate dehydrogenase, pyruvate kinase, triosephosphate isomerase, serum albumin (bovine), and pyridoxal 5′-phosphate were purchased from Sigma Chemical Co. The NBD-Cl was obtained from Pierce Chemical Co., the NSM from Eastman Kodak, the [^{3}H]NEM from New England Nuclear, the quinine sulfate from Aldrich Chemical Co., and the Ludox HS-30 colloidal silica from E. I. du Pont de Nemours and Co. All other chemicals were the best available commercial grade, and all solutions were prepared with deionized-distilled water.

Phosphofructokinase. Rabbit muscle phosphofructokinase was obtained from Sigma Chemical Co. as an ammonium sulfate suspension with 4 mM ATP present (lot 126(-9690)). The enzyme concentration was calculated from the ratio of the absorbances at 280 and 260 nm and from the absorbance at 280 nm using an extinction coefficient of 1.02 mL mg^{-1} cm^{-1} (Parmeggiani et al., 1966) for the native enzyme at 280 nm and correcting for ATP absorption. Absorbances were measured with a Zeiss PMQII spectrophotometer.

The specific activity of the enzyme was determined using coupled enzyme reactions as previously described (Ling et al., 1966; Lad et al., 1973). Assays were run under the following conditions: pH 8.0, 33 mM Tris-Cl, 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 2 mM dithiothreitol, 0.20 unit/mL of aldolase, 35 units/mL of triosephosphate isomerase, 3.2 units/mL of α-glycerophosphate dehydrogenase, and 0.03–0.13 μg/mL of phosphofructokinase in a total volume of 3.0 mL. Assays were initiated by the addition of phosphofructokinase, and the reaction velocity was followed by monitoring the change in absorption at 340 nm on a Cary 118 spectrophotometer thermostated at 23 °C.

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received May 10, 1977*. This work was supported by a grant from the National Institutes of Health (GM 13292).

‡ National Institutes of Health Predoctoral Trainee (GM 00834).

[1] Abbreviations used are: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NSM, 4-dimethylamino-4′-maleimidostilbene; [^{3}H]NEM, *N*-ethyl[2-^{3}H]maleimide; PMP, pyridoxamine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.