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Modification of the Reactive Sulfhydryl Group in Phosphofructokinase

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Abstract: Modification of rabbit muscle phosphofructokinase (PFK) (ATP:D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) with α-bromo-4-hydroxy-3-nitroacetophenone (BHNA) led to inactivation of the enzyme. The minimum molar ratio of BHNA to PFK (based on a molecular weight of 380 000) sufficient to abolish most (>93%) of the catalytic activity at pH 8 was 5:1. Under these conditions, one sulfhydryl group per protomer molecular weight of 93 000 was modified, and the reaction of the most rapidly reacting thiol group per protomer with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was abolished. Carbamidomethylation of six thiol groups per protomer resulted in a 61% loss of catalytic activity. Incubation of the partially carbamidomethylated enzyme with a fourfold molar excess of BHNA led to the modification of an additional thiol group per protomer and to an overall loss of 82% of the activity exhibited in a control experiment. Protection against inactivation of PFK by a fourfold molar excess of BHNA at pH 7.00 was afforded in the order: magnesium adenosine 5′-triphosphate (MgATP) > magnesium inosine 5′-triphosphate (MgITP) > ATP > ITP. At pH 8.00, the order was MgATP > ATP > MgITP. Fructose 6-phosphate (F6P), fructose 1,6-diphosphate (FDP), cyclic 3′,5′-adenosine monophosphate (cAMP), adenosine 5′-monophosphate (AMP), and citrate offered no protection. Modification with a fourfold molar excess of BHNA did not denature the enzyme or alter its aggregation state significantly. Incorporation of 1 mol of BHNA per mole of protomer resulted in a 40% quenching of the native enzyme fluorescence. The CD spectrum of this BHNA-modified enzyme showed that the bound chromophore was immobilized. The binding of cAMP to PFK was unaffected by modification with a fourfold molar excess of BHNA, but the binding of ATP at its single detectable site (apparent $K_d = 3.2$ μM) was abolished. Spin-labeling of the most reactive thiol group per protomer with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide abolished its reaction with DTNB and resulted in a 40% loss of catalytic activity. No quenching of the native protein fluorescence accompanied this modification. Because modification of the single very reactive thiol group per protomer of rabbit muscle PFK does not result in complete abolition of the enzyme's catalytic activity, it cannot be an essential active site functional group. While there appears to be an intimate connection between at least one MgATP binding site and this thiol group, the observation that BHNA-modified PFK still exhibits a low level of catalytic activity suggests that the thiol group does not directly bind MgATP. The reactive thiol plays no role in the binding of cAMP. Finally, depending on the modifying reagent, a conformational change may accompany sulfhydryl modification and influence catalytic activity and binding at one or more MgATP sites.

Although phosphofructokinase (PFK) is an important enzyme in the regulation of glycolysis, relatively little is known about the constitution of its active site(s) and about its mechanism of action. In 1967, Paetkau and Lardy[1] showed that sulfhydryl groups were necessary for the catalytic activity of the muscle enzyme. Froede et al.[2] modified sheep heart PFK with the sulfhydryl-specific reagents, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzoate (PCMB), and concluded that the sulfhydryl groups were not at the catalytic site but linked to it by a conformational change. The evidence for the absence of sulfhydryl groups from the active site was twofold: (1) the degree of inactivation was dependent on the sulfhydryl reagent employed, and (2) neither substrate, fructose 6-phosphate (F6P) or adenosine 5′-triphosphate (ATP), protected the enzyme from inactivation by these reagents. No evidence supporting the hypothetical conformational change was presened. On the other hand, Younathan et al.[3] suggested the proximity of thiol groups to the catalytic site of rabbit muscle PFK, since magnesium adenosine 5′-triphosphate (MgATP) and F6P protected the enzyme against inactivation by iodoacetamide, and F6P prevented the modification of two thiol groups per protomer by DTNB.

Hofer[4] argued that in favorable cases modification of one sulfhydryl group per protomer of muscle PFK would abolish the catalytic activity. However, to reach his conclusions, he employed PCMB (which Chapman et al.[5] claimed to be a relatively ineffective inactivator of the enzyme) in conjunction with the reagents *N*-ethylmaleimide (NEM), tetranitromethane, and 1-fluoro-2,4-dinitrobenzene (FDNB), which are less specific for sulfhydryl groups. Kemp[6,7] and Mathias and Kemp[8] related the reactivity of a single thiol group per protomer to the binding of MgATP at a regulatory site on muscle PFK, and Jones et al.[9] showed that the two sites were relatively close, but not close enough for a modifying group on the sulfhydryl to overlap the MgATP binding site. All three proposed that binding to the MgATP site resulted in a conformation change which affected the sulfhydryl reactivity.

As part of a program of research we have undertaken to investigate the mechanism of action of PFK, we wished to explore the contribution of this residue to the enzymic properties. Its significance for catalysis had not been shown conclusively by Hofer[4] and had not been studied at length by other workers. By the same token, its role in binding had been implied but not demonstrated since (1) it had not been shown that modification of this sulfhydryl group affected the binding of MgATP and (2) its role in the binding of other ligands, e.g., nucleotides, had not been excluded. Further, the only structural probe employed to assess the enzyme microenvironment around the sulfhydryl was a spin label, which was utilized primarily to estimate the distance to the MnATP binding site,[9] and not to construct the relationship to the active site. The aggregation state of the enzyme under these conditions, a parameter which may influence binding and catalytic properties greatly, was not determined.

Our approaches to resolve these questions involved, first, a comparison of the effects on the catalytic activity due to the specific modification of the reactive thiol with several different reagents with the goal of establishing the role of this residue in the catalytic mechanism. Next, standard equilibrium determinations were made of the binding of ATP and another representative nucleotide, cyclic 3′,5′-adenosine monophosphate (cAMP), to enzyme modified specifically at the most reactive thiol per protomer. The aggregation state of the enzyme under the modification conditions was also determined. Finally, the introduction onto the enzyme of a new "reporter group", and the application of fluorescence and circular dichroism (CD) spectroscopy, enabled us to develop arguments concerning the relationshi of this reactive sulfhydryl group to the active site.

Experimental Section

Materials. All chemicals and biochemicals used were of the highest commercial purity available. Iodoacetamide was recrystallized from water and washed with hexane immediately before use.[10] *p*-Nitrophenol was recrystallized from benzene. Sodium lauryl sulfate (SDS) was twice recrystallized from ethanol. 2-Propanol was distilled before use, while acetonitrile was distilled twice over P_2O_5, stored at 4 °C, and used within 2 weeks.

Dialysis membrane from Union Carbide was prepared by boiling for 30 min successively in 2% $NaHCO_3$, 10 mM EDTA, and deionized distilled water before storing at 4 °C in 50% (v/v) ethanol–water. Deionized (by passage through a Continental mixed-bed ion exchanger) distilled water was used for all experiments.

Methods. General. Stock solutions of biochemicals were adjusted to pH values affording maximum stability (pH 8–9 for ATP; 0.01 M Tris–Cl, pH 8, or 1% $NaHCO_3$ for NADH; and pH 7 for AMP, cyclic 3′,5′-AMP (cAMP), ITP, F6P, and FDP)[11] and stored at 4 °C. They were generally used within 1 week. ATP, F6P, and NADH, all components in the standard PFK coupled assay system (in the forward direction), were determined by making them the limiting components in that system,[12] as was ITP, an alternative substrate in the system. FDP, a substrate for aldolase, was determined in the same system minus Mg^{2+}, ATP, and F6P. The coupling (auxiliary) enzymes aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase were minimally determined by increasing their concentrations in the PFK assay system to ensure that the PFK reaction was really rate limiting. AMP and cAMP concentrations were determined spectroscopically from an extinction coefficient of 1.54×10^4 M^{-1} cm^{-1} (259 nm, pH 7).[13]

Syntheses. α-Bromo-4-hydroxy-3-nitroacetophenone (BHNA) was synthesized following the procedure of Sipos and Szabo.[14] Two recrystallizations from chloroform–petroleum ether (bp 60–90 °C) yielded yellow needles [mp 91.5–92.0 °C (lit.[14] mp 93 °C)].

***N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide** was prepared by the improved method of McConnell et al.[15] Recrystallization from toluene–petroleum ether and benzene–hexane yielded orange crystals [mp 114.6–115.6 °C (lit.[15] mp 114–117 °C)].

Isolation and Purification of Phosphofructokinase from Rabbit Skeletal Muscle. Phosphofructokinase was extracted and purified from rabbit skeletal muscle by modifying the procedure of Kemp and Forest.[16] After dialysis for 24 h against 50 mM β-glycerophosphate, 2 mM ATP, pH 7.2, containing ammonium sulfate at 32% of saturation, the enzyme suspension was dialyzed against 0.1 M Tris–phosphate, 0.2 mM EDTA, pH 8, and fractionated on a column of DEAE-cellulose, following the procedure of Ling et al.[12]

The enzyme, which had not been crystallized or otherwise exposed to ATP after DEAE-cellulose fractionation, was stored at 4 °C as a 50% ammonium sulfate suspension in 50 mM β-glycerophosphate, 2 mM EDTA, pH 8.0, containing 0.5 mM dithiothreitol and 1 mM FDP and used for up to 2 months.[17] During this period, the enzyme activity decreased by as much as 20–25%, but no obvious effects of aging (or of the particular preparation) were seen in the properties investigated. Initial specific activity at 25 °C was as high as 125, and usually around 100, units/mg, comparable to that reported in the literature.[18] The yield was generally about 140 mg of purified enzyme from 600 g of muscle. For purified PFK, the A_{279}/A_{259} ratio varied from 1.60 to 1.68 (compared to a maximum ratio of 1.68 reported by Parmeggiani et al.[19] and no charcoal treatment to remove tightly bound nucleotide was deemed necessary.

Measurement of Enzyme Activity. The standard spectrophotometric coupled assay procedure of Ling et al.,[20] modified by Lad et al.,[21] was employed to measure enzyme activity at pH 8.0 and 25 °C. The catalytic activity of PFK was determined from the rate of oxidation of NADH and monitored by the decrease in absorbance at 340 nm, using an extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} for NADH,[22] a unit of enzyme activity being defined as the capacity to oxidize 1 μmol of NADH per minute under specified conditions. Before each day's assays, the three auxiliary enzymes were made up in 0.01 M Tris–Cl, pH 8.0, containing 2 mg/ml of BSA and 1 mM EDTA, and dialyzed at 4 °C against 100 vol of the same buffer minus BSA to remove ammonium sulfate. The assay solution (pH 8.0) contained 33 mM Tris–Cl, 50 mM KCl, 5 mM $MgSO_4$, 2 mM disodium ATP, 2 mM dipotassium F6P, 1 mM dithiothreitol, 0.08–0.1 μg/ml of PFK, 0.56 units/ml of aldolase, 57 units/ml of triosephosphate isomerase, 3.5 units/ml of α-glycerophosphate dehydrogenase, and 0.1 mM NADH. PFK was diluted at 4 °C in 0.1 M KH_2PO_4, 1 mM EDTA, pH 8, containing 2 mM dithiothreitol, and 1 mM F6P. The enzyme concentration range was chosen to keep initial velocity proportional to the PFK concentration.[23] After the rate of NADH oxidation in the absence of PFK was recorded, reaction was initiated by the addition of PFK and observed spectrophotometrically at 25 °C. Virtually identical results were obtained when the activity was assayed directly using the pH-Stat assay of Dyson and Noltmann.[24]

At pH 6.9 and 25 °C, purified PFK exhibited kinetic behavior qualitatively similar to that observed by Hofer and Pette,[25] with respect to inhibition by ATP, and activation by F6P, cAMP, and AMP. The spectrophotometric coupled assay procedure followed was identical with that already described except that 12 mM PIPES buffer replaced 33 mM Tris–Cl, the typical F6P concentration was now 0.2 mM, and appropriate concentrations (5–500 μM) of cAMP or AMP were included in the assay solutions.

Measurement of Protein Concentration. During purification, up to the DEAE-cellulose step, protein was determined by the biuret method of Gornall et al.[26] For applications involving low concentrations of purified PFK or modified PFK, the Lowry method[27] was employed. Bovine serum albumin (BSA) was used as a standard for all colorimetric protein determinations. Otherwise, protein concentration was determined spectrophotometrically using $\epsilon_{279}^{1cm} = 1.02$

mg^{-1} ml (pH 7.2)[19] or $\epsilon_{290}^{1cm} = 1.09$ mg^{-1} ml (0.1 N NaOH).[28]

Determination of Sulfhydryl Content. The total thiol content of PFK was determined by adding an appropriate aliquot of enzyme solution to 2–3 ml of either 50 mM glycylglycine, 0.2 mM EDTA, pH 8, or 25 mM glycylglycine, 0.25 mM EDTA, pH 8, containing sufficient SDS to make the final concentration after all additions 3 mM. After a few minutes incubation at 25 °C excess DTNB was added, and the reaction was followed at 25 °C. An extinction coefficient of 13.6×10^3 M^{-1} cm^{-1} at 412 nm[16,29] or 11.6×10^3 M^{-1} cm^{-1} at 420 nm[30] was taken for the thionitrobenzoate ion. Stock solutions of DTNB concentrations determined with excess dithiothreitol were usually prepared in 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, kept frozen and used within 1–2 weeks. To determine the concentration of a single rapidly reacting thiol group per protomer molecular weight of 93 000, either the procedure of Kemp[7] or that of Jones et al.[30] was employed. The thiol content of glutathione and *N*-acetyl-L-cysteine solutions was determined by titration with excess DTNB.

Modification of Phosphofructokinase with BHNA. Stock solutions of BHNA were prepared in acetonitrile in foil-covered stoppered Pyrex volumetric flasks, stored at 4 °C, and generally used within 2 weeks. The concentration of BHNA was measured spectroscopically (an extinction coefficient of 1.737×10^4 M^{-1} cm^{-1} at 2.5% (v/v) CH_3CN, 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, was measured) and by determination of the loss of free sulfhydryl groups after incubation with a onefold excess of glutathione or *N*-acetyl-L-cysteine in 3 ml of 0.1 M KH_2PO_4, 1 mM EDTA, pH 8, at 25 °C for 1 h.[31]

To carry out the modification of PFK, an appropriate aliquot of stock enzyme suspension was dialyzed under nitrogen at 4 °C against 100 vol of 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2, containing 2 mM dithiothreitol and 1 mM FDP, 100 vol of the same buffer without dithiothreitol or FDP (two changes), and 100 vol of the reaction buffer, either 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, or 50 mM β-glycerophosphate, 2 mM EDTA, pH 8.0. The final dialyzate was diluted to the desired concentration with flushed reaction buffer, passed through a Millipore filter, and allowed to warm to room temperature, after which the pH, protein concentration, and enzyme activity were quickly determined. An appropriate dilution of stock BHNA in acetonitrile was made, and aliquots of acetonitrile and BHNA solution were added to the control and reacting solutions, respectively. A blank solution lacking enzyme was also treated with BHNA. The final volume percentage of acetonitrile was either 2.4 or 0.83 (no difference in properties was observed). After 20 min at room temperature, all three solutions were transferred to a 4 °C cold room. Depending on the purpose, the control and modified solutions were then either dialyzed or passed through Sephadex G-25 (Fine) columns (bed volume was 10–12 times the sample volume) to remove unreacted BHNA or immediately assayed for enzyme activity at pH 8.0 and thiol content. Dialysis and gel filtration (the final buffer was either 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2, or 50 mM β-glycerophosphate, 2 mM EDTA, pH 8.0) were accompanied by continuous flushing with nitrogen. The enzyme concentration employed in the modification reaction was usually 1–2 mg/ml (2.6–5.2 μM, based on a molecular weight of 380 000).

The effect of compounds on the inactivation of PFK by BHNA was tested by incubation with the enzyme for 10–15 min at room temperature immediately before carrying out the activity assays. When Mg^{2+} was required, it was added in 6 mM excess with respect to the nucleotide (ATP or ITP) to force essentially complete complexation, based on a formation constant of at least 15 000 M^{-1}.[32-34] The control enzyme was also preincubated with the compound. Compounds which were substrates in the PFK assay or auxiliary enzyme system were determined from their stock solutions (which were generally made up in the reaction buffer) immediately after the control and modified enzyme solutions were assayed.

The extent of reagent incorporation during modification was estimated as follows. Control, modified, and blank solutions which were not freed of unbound reagent were made at least 50% (v/v) in acetonitrile, completely precipitating the enzyme. The concentration of incorporated BHNA was obtained by difference from the supernatant concentrations. To estimate the error due to coprecipitation of unbound reagent, the same procedure was carried through with reagent which had been treated for 5 h at 100 °C in 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, and which then did not diminish the sulfhydryl content of *N*-acetyl-L-cysteine or glutathione. In a second procedure, an appropriate aliquot of modified enzyme freed of unbound reagent was diluted at room temperature into 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2, containing 3 mM SDS to denature the enzyme (see Methods, above). However, the spectrum of the modified enzyme with SDS present (which showed a peak due to bound chromophore at 315 nm), while differing from that of modified enzyme in the absence of SDS (which showed a broad peak from 317–323 nm), also differed from that of free BHNA in either system (which showed a peak at 320 nm), and therefore the method was not used.

Difference spectral measurements revealed little change when BHNA reacted with PFK. Therefore, the concentration of modified enzyme was estimated either by assuming that in 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2, or 50 mM β-glycerophosphate, 2 mM EDTA, pH 8.0, the spectrum of modified enzyme was a simple sum of the enzyme and BHNA spectra and utilizing the A_{320}/A_{279} of 2.23 for free BHNA and the A_{320} of ~0 for free enzyme in these buffers, or by the Lowry method.[27]

Spin Labeling of Phosphofructokinase. Modification of the most reactive (toward DTNB) thiol group of PFK per protomer weight of 93 000 with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide was carried out as described by Jones et al.[30] Since only a small concentration of spin label was required, a stock solution in water was prepared immediately beforehand. The pH was maintained at 7.2 with a pH-Stat (titrant, 0.02 N NaOH). A control sample was incubated under the same conditions. The reaction was followed by periodically titrating aliquots of the control and modified enzyme solutions with DTNB in the presence of 1 mM F6P (see Methods, above). When the difference between the two titration curves corresponded to 0.9 thiol groups per protomer, the control and modified enzyme solutions were dialyzed against a suitable buffer at 4 °C and assayed at pH 8.0.

Carbamidomethylation of Phosphofructokinase. Alkylation of the enzyme with iodoacetamide was effected by two different methods. To carbamidomethylate PFK partially,[3] the enzyme, at a concentration of about 2 mg/ml (5.3 μM), was dialyzed immediately beforehand against 50 mM glycylglycine, 0.2 mM EDTA, pH 8.0, containing 2 mM dithiothreitol at 4 °C, passed through a Millipore filter, and brought to room temperature. An amount of iodoacetamide equivalent to 1 mM in excess of the total thiol concentration (2 mol of -SH per 1 mol of dithiothreitol) was added, and the solution was incubated for 1 h in the dark at room temperature. The control and modified enzyme solutions were dialyzed against a convenient buffer at 4 °C and assayed at pH 8.0. To carbamidomethylate PFK quantitatively,[35] the enzyme was dialyzed against 0.1 M Tris–phosphate, pH 8.0 at 4 °C, brought to room temperature and incubated for 12 h, under nitrogen, in the same buffer containing 0.01 M dithiothreitol and 6.7 M guanidine hydrochloride. A 1.5 M excess (2.5 times the total thiol concentration) of iodoacetamide was added, and the solution was incubated for a further 1 h at room temperature in the dark. The enzyme was then dialyzed at 4 °C vs. water (the enzyme precipitated).

Circular Dichroic (CD) and Fluorescence Spectra. PFK samples were dialyzed against 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2, or 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, at 4 °C, passed through Millipore filters, and brought to room temperature. Samples were assayed for activity at pH 8.0 before and after spectrophotometric measurements.

CD spectra at 25 °C were obtained using a Cary 60 spectrophotometer with continuous nitrogen flushing of the system. Enzyme concentration varied from 0.65 to 1.7 mg/ml. The data were plotted as mean residue ellipticity (θ), in deg cm^2 $dmol^{-1}$ [35] vs. λ (nm), each point representing an average of three scans of sample and blank.

Fluorescence spectra were obtained at 25 °C under nitrogen using an Aminco-Bowman ratio spectrophotofluorometer equipped with an X–Y recorder or connected to a Honeywell strip-chart recorder. For emission spectra, slitwidths were 4 mm (excitation and phototube entrance slits) and 1 mm (emission slit). Enzyme concentration varied from 0.17 to 2.9 mg/ml (0.45–7.6 μM).

Analytical Ultracentrifugation. PFK was dialyzed against 50 mM β-glycerophosphate, 2 mM EDTA, pH 8.0, or 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2, at 4 °C and centrifuged in a conventional preparatory centrifuge before being brought to room temperature. Protein concentration varied from 3 to 6 mg/ml. The enzyme was assayed at pH 8.0 before and after each experiment.

Sedimentation velocity profiles were obtained using a Beckman-Spinco Model E analytical ultracentrifuge with An-D rotor, single sector cell (12-mm centerpiece, 4° sector), and Schlieren optics. Run

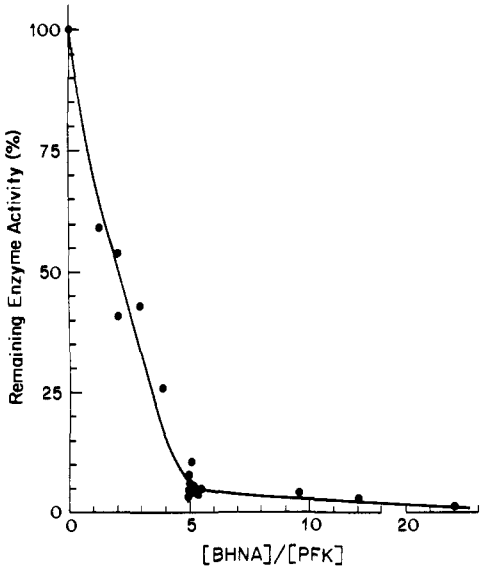


Figure 1. Residual enzyme activity after BHNA modification of PFK. The enzyme, at a concentration of 1-2 mg/ml (2.6-5.2 μM, based on a molecular weight of 380 000), was incubated with BHNA at room temperature (23 °C) for 20 min in 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, containing 2.4% (v/v) acetonitrile. Excess BHNA was removed by dialysis or passage through a Sephadex G-25 (Fine) column, and enzyme activity was determined at pH 8 using the coupled assay system described in Methods. The solid line represents a best fit (by eye) to the data.

temperatures were 21-23 °C and run speeds 40 000 or 56 000 rpm. Photographs were taken at 2- or 4-min intervals at a diaphragm angle of 60° on Kodak spectroscopic plates (Type II-G). Schlieren peak distance to the reference line was measured at least three times using a Nikon microcomparator equipped with a Goertner digitizer. Sedimentation constants were determined graphically following the procedure of Schachman.[37]

Binding Studies by Equilibrium Dialysis. The binding of [8-^{3}H]-cAMP to PFK was measured following the procedure of Setlow and Mansour.[38] PFK was dialyzed as for BHNA modification, except that the final buffer was 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0. Protein concentration ranged from 0.35 to 0.55 mg/ml (3.8-5.9 μM, based on an approximate protomer molecular weight of 93 000). Stock solutions of radioactive ligand were prepared in the same buffer in an ice-water bath and brought to room temperature just before the experiment. Total initial cAMP concentration was varied from 0.5 to 25 μM. Equilibrium dialysis cells with a capacity of 200 μl were constructed following the basic design of Englund et al.[39] The cells were shaken (on an automatic shaker) at a temperature of 23 °C for 4 or 8 h. A control experiment performed without enzyme showed that equilibrium had been reached in each case. Enzyme activity at pH 8.0 and protein concentration (by the Lowry method)[27] were determined. A 100-μl aliquot of each final dialysate was added to 10 ml of Bray's solution[40] and counted in a Packard Tri-Carb liquid scintillation counter. The apparent sample counting rate was always 1000-100 000 cpm. Samples were counted for a total of at least 3 min each. Stock ligand concentration was determined spectroscopically. The binding of [8-^{3}H]-ATP was measured similarly, except that the enzyme concentration was 0.54-0.55 mg/ml (5.8 μM) and the initial nucleotide concentration 1.3-13 μM. Stock ATP concentration was determined spectroscopically and enzymatically. Results were calculated by the method of Scatchard[41] (eq 1)

$$\frac{\bar{n}}{c} = -(1/K_d)(\bar{n} - n) \quad (1)$$

where $\bar{n}$ is the average moles of ligand bound per mole of protein (the number of moles of bound ligand is obtained from the difference in radioactivity between the two sides of the dialysis cell), c is the free ligand concentration (obtained from the radioactivity on the ligand side of the cell), K_d is the dissociation constant for the ligand, and n is the number of independent, identical binding sites on the enzyme. A plot of $\bar{n}/c$ vs. $\bar{n}$ yields K_d from the slope and n from the x intercept. Least-squares fits with standard deviations were calculated.

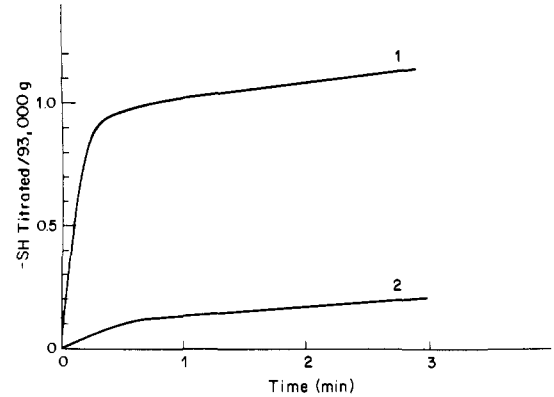


Figure 2. The titration of native and BHNA-modified PFK with DTNB in 25 mM β-glycerophosphate, 1 mM EDTA, 0.08 mM ATP, 0.4 mM F6P, pH 7.2, at 25 °C. Enzyme concentration was 6.57 μM (based on a molecular weight of 93 000); DTNB concentration, 10.4 μM. The modification of PFK with a fourfold molar excess of BHNA was carried out as described in the legend for Figure 1: curve 1, native PFK; curve 2, modified PFK.

Results

Modification of Phosphofructokinase by BHNA. Modification of PFK with BHNA led to inactivation of the enzyme, as shown in Figure 1. The minimum molar ratio of BHNA to PFK (based on a molecular weight of 380 000)[18] sufficient to abolish most (>93%) of the catalytic activity at pH 8 was 5:1, which corresponds to about 1 mol of BHNA per protomer weight of 93 000.[28] The apparent extent of inactivation was the same in each of the buffers employed. Extending the period of incubation with BHNA to 3 h did not alter the extent of inactivation.

Site and Stoichiometry of BHNA Incorporation. Since sulfhydryl groups are known to be important for the catalytic activity of PFK,[1] and since PFK possesses an extremely reactive thiol group per protomer molecular weight of 93 000,[16] it was expected and found that the inactivation of PFK by a 5:1 molar ratio of BHNA corresponded to the alkylation of a single thiol group per protomer, analogously to the inactivations of papain[42] and transglutaminase[43] by equimolar concentrations of BHNA. Inactivation of PFK by BHNA was accompanied by a loss of sulfhydryl content, which appeared to be linearly proportional to the ratio of BHNA to enzyme.

The thiol group could not have been simply "buried" upon modification of another residue because the denaturing conditions of the DTNB titration would have reexposed it. Thus, reaction of a single thiol group per protomer with BHNA led to a >93% inactivation of PFK. In support of this conclusion, it was found that modification of PFK with a fourfold molar excess of BHNA abolished the reaction with DTNB of the most rapidly reacting thiol group per protomer (Figure 2).

To show that incubation with a fourfold molar excess of BHNA led to the incorporation of 1 mol of reagent per protomer, bound reagent was estimated by difference after precipitation of the modified enzyme. Modification with [BHNA]/[PFK] = 6.3, under the above conditions, led to the binding of 0.92-1.3 mol per protomer, while modification with [BHNA]/[PFK] = 5.4 (7% of control activity remaining) resulted in 0.80-1.1 mol of BHNA being bound.

An interesting result was obtained when the enzyme was first partially carbamidomethylated with iodoacetamide and then incubated with a fourfold molar excess of BHNA. Carbamidomethylation resulted in a 61% loss of control activity, corresponding to the alkylation of six thiol groups per protomer. However, modification of the carbamidomethylated enzyme with BHNA led to a further loss of 53% of its catalytic activity at pH 8 (an overall loss of 82% of control activity).

Table I. Protection against Inactivation of PFK by BHNA[a] Afforded by Various Compounds[b]

Compd	Concn, mM	pH	Remaining enzyme act., %[c]	Remaining enzyme act. in absence of compd, %[c]
MgATP	0.86	7.0	87	6.5
	0.82	8.0	49	6.0
	2.7	8.0	97	4.4
ATP	1.4	7.0	39	11
	2.4	7.0	46	6.1
	2.3	8.0	42	7.7
MgITP	0.73	7.0	18	3.9
	2.2	7.0	53	5.2
	2.0	8.0	23	6.1
ITP	2.2	7.0	19	4.4

[a] Modification of PFK with a fourfold molar excess of BHNA was effected as described in the legend of Figure 1, in a buffer of 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, or 50 mM β-glycerophosphate, 2 mM EDTA, pH 8.0. [b] The enzyme was preincubated with each compound for 10–15 min at room temperature (23 °C) before modification. When Mg^{2+} was required, a 6 mM excess was employed. [c] Expressed as a percent of the appropriate control activity.

Titration with DTNB showed that an additional (a seventh) thiol group per protomer had been modified by BHNA.

Protection of Phosphofructokinase against Inactivation by BHNA. In order to determine if BHNA was active-site directed, as it is in the case of papain[42] and transglutaminase,[43] the effect of preincubating PFK with relatively high concentrations of substrates prior to reaction with a fourfold molar excess of BHNA was tested. It can be seen from Table I that MgATP was by far the most effective at protecting the enzyme from inactivation; even at higher concentrations, MgITP (which is a somewhat less effective substrate for PFK)[44] and ATP alone afforded less protection. The increased protection afforded in the presence of Mg^{2+} is in accord with the much stronger binding of MgATP and MgITP to PFK than that of ATP and ITP, respectively.[17,32] ITP offered only slight protection, while the nucleotides cAMP and AMP, the other substrate, F6P, the product, FDP, and citrate, an allosteric inhibitor of PFK,[21] did not protect at all against the loss of activity. At pH 8.0, the degree of protection lent by fixed concentrations of MgATP and MgITP, but not ATP, decreased markedly compared to that afforded at pH 7.0.

Conformation and Aggregation State of PFK Following Modification with BHNA. To be a useful, structural, or mechanistic probe of an enzyme, it is necessary that a reagent not denature the enzyme or alter its aggregation state, respectively. Sedimentation velocity runs were made and the Schlieren patterns of native and modified enzyme compared. The observed pattern of three peaks was in qualitative agreement with that reported in the literature.[12,19,45,46] For PFK modified by a fourfold excess of BHNA the sedimentation coefficients were almost identical with those for the native enzyme. The ratios of peak areas, which were estimated by projecting the magnified plate images onto graph paper, were also nearly unchanged.

The CD spectra of native and BHNA-modified enzyme were compared for evidence of a difference in conformation. Figure 3 shows that the CD spectra of native PFK and PFK modified with a fourfold molar excess of BHNA were virtually identical in the region 200–250 nm. The retention of the strongly negative band in this region suggested that the rigid protein "backbone" structure had been preserved. The difference in the region around 290 nm, which corresponds to a difference in the environment around tryptophan,[47] probably indicated a local conformational change. Above 300 nm, where

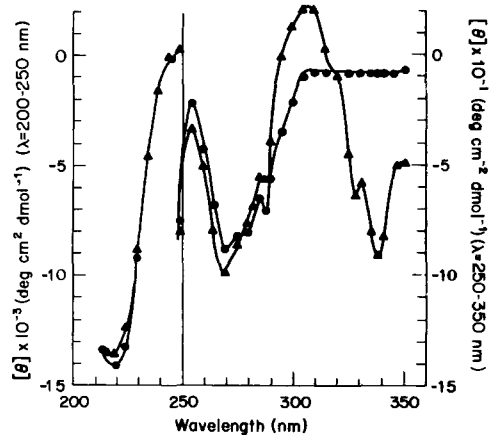


Figure 3. CD spectra of native PFK and PFK modified with a fourfold molar excess of BHNA, as described in the legend for Figure 1. Both were dialyzed against 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2. Control (●) and modified (▲) enzyme concentrations were 1.7 and 0.65 mg/ml, respectively. Spectra were recorded on a Cary 60 spectropolarimeter at a temperature of about 25 °C.

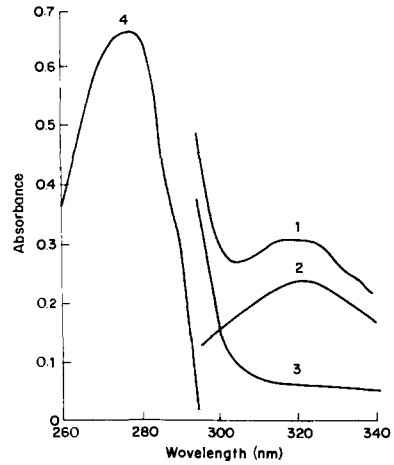


Figure 4. The uv absorption spectra of BHNA-modified PFK and free BHNA. PFK was modified for 20 min at 23 °C by a fourfold molar excess of BHNA as described in the legend for Figure 1 and dialyzed against 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.0, to remove excess reagent. Spectra were measured vs. the same buffer on a Cary 15 spectrophotometer at 25 °C: curve 1, the bound chromophore peak; curve 2, free BHNA at a concentration of 13.7 μM; curve 3, the control enzyme spectrum in the region of the bound chromophore [enzyme concentration was 1.12 mg/ml (2.95 μM)]; curve 4, the modified enzyme peak, offset downscale by 0.5 OD units [enzyme concentration was 1.04 mg/ml (2.74 μM)].

the native enzyme showed no CD spectrum, the modified enzyme showed a strong negative band (340 nm), corresponding to a bound, relatively immobilized BHNA chromophore.

In this connection it should be noted that although the uv difference spectrum of BHNA-modified PFK showed a broad peak from 317 to 323 nm, corresponding to the bound chromophore, in contrast to the sharp peak at 320 nm for free BHNA in buffer, no significant change ($\Delta\epsilon$ appeared to be $<1000\ M^{-1}\ cm^{-1}$) in absorbance large enough to be measured accurately and reproducibly was noted when the reaction of enzyme with a fourfold molar excess of BHNA was monitored over this wavelength range. Figure 4 shows the uv spectrum of BHNA-modified PFK compared to those of native PFK and free BHNA.

Quenching of Native PFK Fluorescence by Modification with BHNA. In view of the apparent change in the environment of tryptophan resulting from modification of a single reactive sulfhydryl group per protomer of PFK with BHNA, it was of

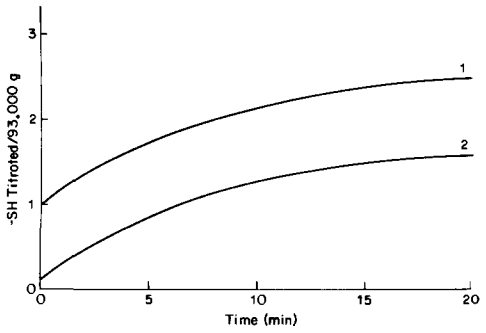


Figure 5. The titration of native and spin-labeled PFK with DTNB at 25 °C in 0.05 M Tris–phosphate, 1 mM EDTA, 1 mM F6P, pH 6.65. Enzyme concentration was 3.7 μM (based on a molecular weight of 93 000); DTNB concentration, 42 μM. The spin labeling of PFK was carried out as described in Methods: curve 1, native PFK; curve 2, modified PFK.

Table II. Quenching of Native PFK Fluorescence[a] by Modification with BHNA[b]

Enzyme concn, μM (1 mg/ml = 2.6 μM)	Excitation wavelength, nm	% quenching of native fluorescence
0.45	260	43
	280	40
	300	37
	320	3
1.2[c]	260	42
	270	40
	280	42
	290	43
	300	42
1.7	260	52
	280	53
	300	56
	320	35
4.5	260	64
	280	65
	300	66
	320	60

[a] Spectra were recorded on an Aminco-Bowman ratio spectrophotofluorometer at 25 °C. Relative fluorescence intensity was measured against a blank lacking enzyme at the emission maximum of 340 nm. [b] PFK was modified with a fourfold molar excess of BHNA as described in the legend for Figure 1 and dialyzed against 25 mM β-glycerophosphate, 1 mM EDTA, pH 7.2. [c] The buffer employed was 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0.

interest to compare the fluorescence spectra of modified and native enzyme. The relative fluorescence intensity of native PFK at its emission maximum of 340 nm decreased when the enzyme was modified with BHNA, though the wavelength of maximum emission was unchanged. Table II gives the extent of quenching over a tenfold range of protein concentration, for several different excitation wavelengths. It is apparent that the degree of quenching was roughly independent of the excitation wavelength, except at an excitation wavelength of 320 nm, where it was slightly lower, and that it increased with increasing protein (and therefore BHNA) concentration. It should also be noted that in this protein concentration range, fluorescence intensity was not linearly proportional to concentration. Since PFK contains 13 tryptophans per protomer,[28] fluorescence intensity would not be expected to be linear with concentration above protein concentrations of about 0.2 μM (0.0076 mg/ml).[48,49]

Modification of PFK by Specific Spin Labeling. When PFK was specifically modified with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide at its most reactive sulfhydryl group per protomer, only 38% of the catalytic activity at pH 8 was lost. Figure 5 shows the time course of DTNB titrations of native and 90% spin-labeled PFK in the presence of 1 mM F6P. It can be seen that the reaction with DTNB of a single rapidly reacting thiol group per protomer of molecular weight 93 000 was abolished by the modification, the same result found for the modification of PFK by a fourfold molar excess of BHNA (Figure 2). However, the fluorescence spectra (excitation wavelength, 279 nm; emission maximum, 340 nm) of native and spin-labeled PFK at a concentration of 0.094 mg/ml (0.25 μM) in 0.1 M KH_2PO_4, 1 mM EDTA, pH 8.0, containing 1 mM F6P and 2 mM dithiothreitol, were identical (the protein fluorescence was not quenched), in contrast to the case for native and BHNA-modified enzyme.

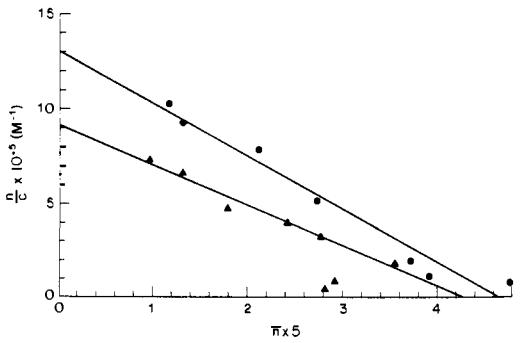


Figure 6. Scatchard plots of the binding of cAMP to BHNA-modified and native PFK. n is the number of moles of ligand bound per 93 000 g of enzyme and c is the free ligand concentration. PFK was modified by a fourfold molar excess of BHNA as described in the legend for Figure 1 and dialyzed against 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0. For the equilibrium dialysis experiments at 23 °C, enzyme concentration varied from 4.5 to 5.5 μM, based on a molecular weight of 93 000. Initial ligand concentration varied from 0.61 to 15 μM. The solid lines are least-squares best fits to the data: (▲) control enzyme; (●) modified enzyme.

Table III. Binding of cAMP by BHNA-Modified and Native PFK[a]

	K_d, μM[b]	n[c]
Modified PFK	0.72 ± 0.02	0.94 ± 0.22
Native PFK	0.93 ± 0.02	0.86 ± 0.20

[a] The procedures for modification of PFK with a fourfold molar excess of BHNA and determination of binding affinities by equilibrium dialysis have been outlined in the legend for Figure 6. [b] Binding constants were obtained from a least-squares analysis of the Scatchard plots shown in Figure 6. The indicated error limits correspond to one standard deviation in the slope and intercept. K_d is the apparent dissociation constant. [c] The value of n, the apparent number of identical binding sites per 93 000 g of enzyme, is based on initial enzyme concentration. Since the enzyme concentrations determined by the Lowry method after each binding experiment were sometimes up to 10% lower than their initial values, probably due to membrane adsorption, this value should be revised slightly upward. The value of K_d is unaffected by this consideration.

Binding of cAMP and ATP to BHNA-Modified PFK. The role of the reactive sulfhydryl group per protomer in binding was further investigated in hopes of confirming its intimate relationship with an ATP binding site. We have seen that cAMP did not prevent inactivation of PFK by BHNA. Scatchard plots[41] of the data obtained from equilibrium dialysis measurements of cAMP binding to modified and native PFK are presented in Figure 6. The binding constants are listed in Table III. Equilibrium dialysis was chosen in preference to the column equilibration technique[50] because it allowed a binding determination at a fixed enzyme concentration.[38] It is apparent

Table IV. Binding of ATP by BHNA-Modified and Native PFK[a]

	K_d, μM[b]	n[c]
Modified PFK	∞	0
Native PFK	3.2	0.75

[a] The procedures for modification of PFK with a fourfold molar excess of BHNA and determination of binding affinities by equilibrium dialysis have been described in the legend for Figure 7. [b] K_d is the apparent dissociation constant. [c] See footnote *c* in Table III.

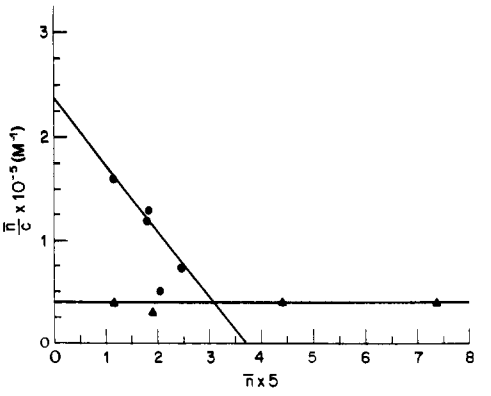


Figure 7. Scatchard plots of the binding of ATP to BHNA-modified and native PFK. The labeling of the axes and the experimental procedure are described in the legend for Figure 6, except that the modified and native enzyme concentrations were 5.4 and 5.5 μM, respectively, and the initial ligand concentration varied from 1.3 to 13 μM. The solid lines represent best fits (by eye) to the data: (▲) modified enzyme; (●) control enzyme.

that within experimental error modification did not affect (or perhaps slightly enhanced) the binding of cAMP to PFK. Native and modified enzyme bound about 1 mol of cAMP per 93 000 g with a dissociation constant of 0.8 μM. Comparable binding constants for native PFK from the literature are K_d = 0.6 μM and n = 1[51] obtained using the column equilibration method. As seen in Table I, however, ATP did afford protection against inactivation by BHNA. When ATP binding to PFK was determined under similar conditions, it was found that BHNA-modified enzyme did not bind the substrate. The Scatchard plots for ATP binding are shown in Figure 7, and the binding constants are collated in Table IV. Within experimental error it appeared that the control enzyme bound 1 mol at ATP per 93 000 g with a dissociation constant of 3.2 μM. Increasing the PFK concentration in the ATP equilibrium dialysis experiments led to an apparent reduction in n, the number of identical binding sites per 93 000 g of enzyme.

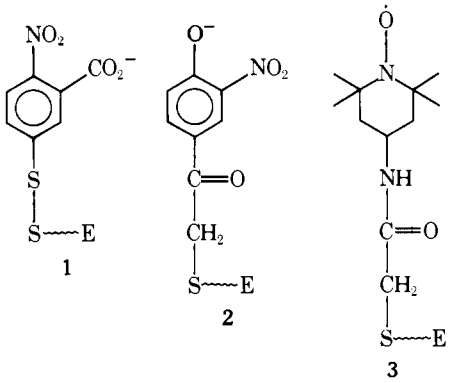
Discussion and Conclusions

Incubation of rabbit muscle PFK with a fourfold molar excess of BHNA (based on a molecular weight of 380 000) led to the rapid loss of over 93% of the enzyme's catalytic activity at pH 8 and the modification of one cysteine group per protomer molecular weight of 93 000. (The solvolysis of free BHNA in the absence of enzyme was quite slow, with a half-life of over 1 day.)

On a basis of the degree of inactivation effected per mole of modifying reagent either employed or incorporated, BHNA was a uniquely effective sulfhydryl-specific inactivator of PFK, as illustrated by the following comparisons. Hofer[4] reported that incubation of PCMB with an equal PFK protomer concentration abolished over 99% of the enzymatic activity and concluded that in favorable cases, a single sulfhydryl group per protomer was involved in the catalytic mechanism of rabbit muscle PFK, although other amino acid residues were also involved. However, Chapman et al.,[5] using different modification conditions, found that a large excess of PCMB was required to inactivate the enzyme completely and that in their system DTNB was a more effective inactivator of the enzyme than PCMB.

Bloxham et al.[18] showed that the 6-mercapto derivative of ATP, sRTP, which is a good substrate of rabbit muscle PFK at low concentrations (the apparent K_m is 10^{-4} M at [F6P] = 5×10^{-5} M, similar to the apparent K_m of ATP under these conditions, 4×10^{-5} M), completely inactivated the enzyme, but only at much higher concentrations. For example, a 2000-fold excess was required to achieve 95% inactivation. sRTP (6 mol) was incorporated (by the formation of disulfide linkages) per mole of enzyme under these conditions.

Kemp and Forest[16] reported that modification of four sulfhydryl groups per protomer with DTNB, the other sulfhydryl-specific reagent reported to be an effective inactivator of rabbit muscle PFK, resulted in only a 91% loss of catalytic activity. Indeed, for 1 mol incorporated per mole of protomer, DTNB caused only a 68% loss of catalytic activity, whereas BHNA effected a loss of over 93%. In both enzyme-bound thionitrobenzoate (**1**) and BHNA (**2**) a negatively charged group is attached to the aromatic ring, although the positions of attachment differ. Figure 2 shows that incorporation of 1 mol of BHNA per PFK protomer blocked the reaction of the most rapidly reacting thiol group per protomer with DTNB, implying that both reagents modified the same thiol group per protomer. The greater efficacy of BHNA as an inactivator of PFK does not appear to be simply related to the increased distance of the bulky ring from the reactive site (three bonds away instead of two) which might have fortuitously placed the ring in a position resulting in increased inaccessibility of the active site to substrate. Figure 5 shows that modification with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (**3**) also blocked the reaction of the most rapidly reacting thiol



group per protomer with DTNB, implying that the spin-label, BHNA, and DTNB were directed to the same site. However, spin labeling the enzyme at this reactive thiol group per protomer resulted in only a 40% loss of catalytic activity, even though the ring is four bonds away from the reactive site. Thus, the orientations of the ring with respect to the reactive sites, due to differences in bond angle, must also be considered and, of course, the effect of the bound group depends on chemical, as well as physical, interactions with its microenvironment.

A further comparison of the effectiveness of BHNA as an inactivator of PFK was suggested by the observation that modification with a fourfold molar excess of the reagent caused a 53% reduction in the catalytic activity of partially carbamidomethylated PFK (six thiol groups per protomer carbamidomethylated) to give an overall loss of 82% of the native enzyme activity. Titration with DTNB showed that a seventh thiol group per protomer had been modified, indicating that the additional loss of activity was not due to modification of

a group other than a cysteine residue. It has already been noted that under the conditions used, carbamidomethylation of even ten thiol groups per protomer leaves a residual activity of 30%.[3] One possible explanation is that the reactive sulfhydryl group per protomer modified by BHNA is not reactive toward iodoacetamide. The sulfhydryl reactivity classification schemes of Kemp and Forest[16] and Hofer[4] did not include reaction with iodoacetamide. Evidence that different sulfhydryl reagents are directed to different sulfhydryl groups has been presented in the case of rabbit muscle creatine kinase.[10] Modification of the available reactive thiol group per protomer by BHNA might lead to only an 82% (instead of >93%) overall activity loss if partial carbamidomethylation limited the extent of a conformational change (which we define operationally in the following paragraph) following BHNA modification. A second possibility is that the reactive thiol group per protomer was modified by iodoacetamide, but the effects of the relatively small, uncharged modifying group on the surrounding enzyme microenvironment were much less than those of BHNA. It must be reemphasized that despite the effectiveness of BHNA as an inactivator of PFK, ca. 7% of the catalytic activity remained after modification of the reactive thiol group per protomer; therefore, the latter is not an essential functional group.

There is evidence that the mechanism of PFK inactivation by a fourfold molar excess of BHNA involves a conformational change leading to increased inaccessibility of the active site to substrate, rather than just a direct blockage of the active site. The lack of a significant shift to longer wavelength in the uv absorption spectrum of bound, compared to free, BHNA (Figure 4) and the slight broadening of the peak in the direction of shorter wavelength indicate that the microenvironment of the attached "reporter group" remains relatively polar.[43] However, the CD spectrum of BHNA-modified enzyme (Figure 3) clearly shows that the bound chromophore (ca. 340 nm) is immobilized, implying binding or at least accommodation of the aromatic portion of the molecule as well. In order for such secondary binding or accommodation to occur, a conformational change might be necessary to bring hydrophobic residues into a more polar locale. The "backbone" and quaternary structure of the enzyme would be preserved (see Results), but bringing a (hydrophobic) tryptophan residue into a more polar environment might lead to quenching of its fluorescence. In fact, significant (at least 40%) quenching of native PFK fluorescence was observed following modification with a fourfold molar excess of BHNA (Table II). No such fluorescence quenching was observed after 90% spin labeling of the same reactive thiol per protomer with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide. Hofer[4] showed that incorporation of 1 mol of PCMB per protomer of rabbit muscle PFK abolished the reaction with DTNB of the most rapidly reacting thiol group per protomer; thus, BHNA, DTNB, PCMB, and the spin label modify the same thiol group per protomer. Unlike BHNA, DTNB, and PCMB, the spin label lacks an aromatic ring. The binding or accommodation of the bulk of the spin label does imply a certain degree of hydrophobicity around the site of the label. However, the increased distance of the bulky ring may in this instance contribute to a relative increase in the mobility of the spin label compared to that of the bound aromatic groups and to a corresponding lowering of the energy associated with a given conformation. Of course, any modification of a relatively exposed thiol group could result in a localized conformational change which might not affect the generally "buried" tryptophan residue(s).

Alternatively, it might be argued that the reactive sulfhydryl group per protomer is near enough to the active site so that the bulky BHNA modifying group blocks access to it, simultaneously quenching the fluorescence of a neighboring tryptophan residue (or residues). The piperidinyl ring of the spin label, an additional bond removed from the reactive sulfhydryl, is presumably farther away from the active site and the tryptophan residue(s). The active site would still not be completely accessible, because of the steric hindrance due to the chain connecting the ring to the reactive sulfhydryl. If we postulate that no conformational change follows modification of the reactive sulfhydryl group per protomer with the spin label, this hypothesis explains why spin labeling still abolishes 40% of the enzyme's catalytic activity. The proximity of the most reactive, and possibly some of the other thiol groups per protomer, to the active site would also explain why carbamidomethylation abolishes 60% of the catalytic activity and why either of the substrates ATP or F6P (2 mM) prevents inactivation.[3] We might also suspect that some of the other thiol groups per protomer are around the active site; for instance, ATP and F6P both slow the rate of titration with DTNB of the two class II thiol groups per protomer of Kemp and Forest.[16] (The reactive thiol per protomer discussed in this work is their class I thiol group.) Actually, the class I thiol group need only be close enough to the active site so that a small perturbation would move the bound BHNA into the position described. The effects of the modification of sulfhydryl groups in PFK probably involve conformational changes in addition to direct blockage of the active site, depending on the reagent. It is noteworthy that the scheme outlined by Koerner et al.[52] indicated that the binding site for F6P was relatively hydrophilic. Although the bound BHNA "reporter" group also seemed to be in a relatively hydrophilic region, F6P did not prevent the inactivation of rabbit muscle PFK by BHNA, indicating that the reactive thiol group per protomer certainly was not right at the active site.

An intimate relationship exists between the reactive sulfhydryl group per protomer modified by a fourfold molar excess of BHNA and at least one ATP binding site on PFK. Not only does ATP protect against the inactivation of PFK by BHNA (Table I), but the incorporation of 1 mol of BHNA per mole of protomer abolishes the binding of at least 1 mol of ATP per protomer (Table IV). No distinction has been made between the MgATP and ATP binding sites. The superior protection against inactivation afforded by MgATP with respect to ATP (Table I) was interpreted in terms of the increased affinity of ATP in the presence of Mg^{2+} for the same binding site. Jones et al.[17] showed that ATP does compete for the Mn(Mg)ATP site. (The K_m values for MgATP and MnATP in the presence of a saturating concentration of F6P are the same.[17]) It was also shown that MnATP binds to the enzyme through a substrate bridge,[17] implying that there is a common binding for MnATP and ATP. In our own ATP binding experiments, we have omitted magnesium ion for two reasons: (1) to compare the results on the same basis with the cAMP binding experiments; and (2) in recognition of the observation that ATP itself was an effective protecting agent between pH 7 and pH 8 against inactivation by BHNA.

The binding of ATP to native PFK deserves some comment. We are not aware of any other determination of ATP binding to PFK employing the method of equilibrium dialysis. Various stoichiometries of binding have been reported. For sheep heart PFK, Lorenson and Mansour,[53] using the column equilibration technique,[50] and thus averaging the binding over a range of enzyme concentrations, indicated that in the absence of other ligands, the number of binding sites for ATP or PFK decreased with increasing enzyme concentration and that at pH 6.7, 25 mM β-glycerophosphate reduced the maximal binding of ATP from 3.6 to 2.4 mol per 100 000 g (the identical K_d values increased from 2 to 7 μM). From the enhancement of water proton longitudinal relaxation rates, Jones et al.[17] found two equivalent (K_d = 240 μM) binding sites for MnATP per 90 000 g of rabbit muscle PFK. The enzyme's aggregation

state under the conditions used by these workers (high protein concentration, 25 mg/ml, in the absence of other ligands) is unknown, and we do not know if the high dissociation constant was a consequence of it. Kemp and Krebs[51] also used the column equilibration method, and they found three distinct sites for ATP on the muscle enzyme with K_d values ranging from 5 to 15 μM (in 25 mM β-glycerophosphate, 25 mM glycylglycine, 1 mM EDTA, 5 mM mercaptoethanol, pH 7.0). The enzyme concentration range was not indicated. Our value of 1 ATP site per 93 000 g with a K_d value of 3.2 μM may correspond to their high affinity site, since our conditions (25 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0) resemble theirs most closely. At the relatively high enzyme concentration we employed (0.55 mg/ml, in the presence of the ligand β-glycerophosphate), the other binding sites may not have been available as a consequence of the state of aggregation. In this regard, we observed that the number of ATP sites per protomer decreased (by a factor of 10) as the enzyme concentration was increased (to 1.9 mg/ml). Another possibility is that the lower affinity sites were not detected under our experimental conditions.

Abolition of the binding of ATP to this site following modification of one sulfhydryl group per protomer with BHNA does not necessarily imply that the sulfhydryl group is at the binding site. In fact, Jones et al.[9] utilized modification of the same reactive thiol group per protomer with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide to map the relative position of the MnATP binding site (the K_d increased from 260 to 480 μM upon modification); the manganese ion was estimated to be 12 Å away from the nitroxide radical. We may then postulate that the inaccessibility of the ATP binding site following modification of the most reactive thiol per protomer with BHNA resulted from the ensuing conformational change as discussed, since the two sites were not close enough for a modifying group on the sulfhydryl residue to overlap the MnATP binding site. It is certainly plausible that the same conformational change could make both the active site and the ATP binding site inaccessible if the two sites are not far apart. In the case of the spin-labeled enzyme, such a conformational change presumably did not occur, and both MnATP sites remained available.

It is not strictly appropriate to speculate whether the ATP binding site per protomer we observed was a catalytic or inhibitory one (using the terminology employed, for example, by Mathias and Kemp[8]), since the binding conditions are not the same as the kinetic assay conditions for catalysis or ATP inhibition at pH 8.0 or 6.9, respectively. We would have expected to detect at least two ATP binding sites per native PFK protomer, corresponding to a catalytic and a regulatory site, based on previous reports of the separability of the two kinds of site for rabbit muscle PFK,[5] and in agreement with the experimental evidence of Jones et al.[17] The binding of MgATP to the inhibitory site decreases as the pH increases from 7.0,[6] paralleling the trend in the effectiveness of MgATP in protecting against inactivation by a fourfold molar excess of BHNA (Table I). However, the pH dependency of the binding of MgATP to the catalytic site has not been reported. MgITP, which was less effective in preventing BHNA inactivation of PFK than MgATP at the same concentration and pH (Table I), is not only a less effective inhibitor of PFK than MgATP,[54] but it is also a poorer substrate for the catalytic reaction.[44] The abolition of >93% of the catalytic activity at pH 8.0 following modification with a fourfold molar excess of BHNA then may have been due to the effective blocking of MgATP from the catalytic site. However, the observation of residual catalytic activity implies some remaining binding of MgATP to the catalytic site. (It seems very unlikely that we had an impurity with PFK activity.) In addition, Kemp[7] reported that modification of the same (class I) thiol group per protomer with DTNB did not change the K_m value for ITP, a noninhibitory substrate,[54] implying that the nucleotide substrate still had access to the catalytic site. Jones et al.[17] and Chapman et al.[5] saw no effect on the allosteric kinetics (i.e., assays performed at pH 6.9 as described in Methods) after modification of the same uniquely reactive thiol group per protomer with spin label and PCMB, respectively. Kemp[7] observed that the only effect of modification of the same sulfhydryl group with DTNB was to decrease the Hill interaction coefficient for F6P. It would be of interest to examine the allosteric kinetics of PFK modified with a fourfold molar excess of BHNA to reaffirm the apparent separability of catalytic and regulatory sites for ATP on rabbit muscle PFK. In our case, the apparent K_d value for either site could have increased upon modification of the enzyme, so that we would not detect any binding. Most evidence in support of that view has been accumulated in studies on sheep heart PFK.[55]

Since cAMP did not protect against inactivation by a fourfold molar excess of BHNA, and modification of the reactive sulfhydryl group per protomer had no effect (or perhaps slightly enhanced the binding of cAMP to PFK), it can be concluded that the cAMP binding site is distinct from the ATP binding site. Such a separability of nucleotide binding sites on rabbit muscle PFK has been reported by Kemp and Krebs[51] and Mathias and Krebs.[8] Further, sRMP, the 6-mercapto analogue of AMP (which competes for the cAMP binding site[51]), inactivates the enzyme by only 50%, compared to the 100% inactivation by sRTP, the corresponding analogue of ATP.[18]

Studies on the enzymatic digestion of BHNA-modified PFK have been initiated in our laboratory with the objective of determining the amino acid sequence in the vicinity of the reactive sulfhydryl group and will be reported in due course.

Acknowledgments. We are grateful to Dr. F. P. Stuart and Mr. R. Ferrara of the Department of Surgery, Pritzker School of Medicine, University of Chicago, for providing us with rabbits and experimental facilities. This research was supported in part by Grant No. GM 19037 from the National Institute of General Medical Sciences (E.T.K.) and by National Institutes of Health Predoctoral Traineeship, GM-424 (K.J.S.).

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