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Interaction of Dinucleotides with Muscle Phosphofructokinase†

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ABSTRACT: The binding of adenosine diphosphoribose (ADPR), NADH, NADPH, NADP, and NAD by rabbit skeletal muscle phosphofructokinase was determined on the basis of their ability to competitively inhibit the binding of adenosine cyclic 3′,5′-phosphate (cAMP) and of their ability to induce a conformational change as indicated by thiol reactivity. The binding affinities of ADPR and the dinucleotides decreased in the order listed above. The dissociation constant for ADPR was about 5 μM as estimated from the inhibition of cAMP binding. Whereas ADPR and the dinucleotides were capable of blocking the reactivity of two thiol groups in a manner similar to that of other adenine nucleotides, ADPR had no effect on the rate of arylation of the most reactive thiol group of phosphofructokinase, a thiol group whose reactivity is thought to be an indicator of the active–inactive protein conformational transition. Furthermore, ADPR was capable of inhibiting the effect of cAMP on the arylation rate of the reactive thiol group. ADPR was not capable of activating the reaction catalyzed by phosphofructokinase, but instead prevented the activation of phosphofructokinase by cAMP in an apparent competitive manner. These results indicate that ADPR, and presumably the other nicotinamide dinucleotides, binds to the adenine nucleotide activating site of phosphofructokinase but is unable to produce the active conformation. On the basis of the relative affinities of phosphofructokinase for 8-azido-cAMP, 8-amino-cAMP, and cAMP, it was deduced that the adenine nucleotide site favors the anti-glycosidic conformation of nucleotide effectors over the syn-glycosidic form. Substitution at the N-6 position of the adenine ring prevents binding to the adenine nucleotides site in contrast to the known binding behavior of adenine nucleotides to the catalytic site.

Rabbit skeletal muscle phosphofructokinase displays a complex regulatory behavior that is the consequence of its interaction with a variety of metabolites at specific binding sites [see Uyeda (1979) for a review]. Three of the binding sites are capable of tightly binding adenine nucleotides: the catalytic site, an inhibitory site, and an activating site (Kemp & Krebs, 1967). The activating site, which shows greatest specificity for adenine mononucleotides, reacts with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, a derivative of adenosine extended from the 5 position of ribose that has been used for the modification of the dinucleotide site of several dehydrogenases (Pettigrew & Frieden, 1978). This observation, coupled with the known binding of phosphofructokinase to blue dextran, a probe for dinucleotide folds (Bohme et al., 1972), suggested a study of the interaction of nicotinamide adenine dinucleotides with phosphofructokinase.

The present study shows that dinucleotides apparently compete with mononucleotides at the activating site of muscle phosphofructokinase. This interaction does not activate the enzyme but instead inhibits the activating action of the mononucleotides.

Materials and Methods

Enzyme Preparation. Phosphofructokinase (twice crystallized) was prepared from fresh rabbit muscle as described by Kemp (1975). The second crystals were collected by centrifugation and dissolved in the indicated buffer. For removal of ammonium sulfate, the phosphofructokinase solution was dialyzed with one change against 100 volumes of the same buffer, at room temperature. When it was necessary to remove bound ATP, the enzyme solution was passed through a charcoal–cellulose (1:1 w/w) column (4.5 × 20 mm). A 280/260 absorbancy ratio greater than 1.6 was taken as an indication that ATP had been essentially removed from the protein (Parmeggiani et al., 1966).

Binding Experiments. The fast-flow equilibrium dialysis method of Colowick & Womack (1969) was used to determine the dissociation constant for adenosine cyclic 3′,5′-phosphate (cAMP)[1] and for competitive binding assays. The cylindrical

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[1] Abbreviations used: ADPR, adenosine diphosphoribose; NMN, nicotinamide mononucleotide; cAMP, adenosine cyclic 3′,5′-phosphate; DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; IMP, inosine 5′-phosphate; $(Nbs)_2$, 5,5′-dithiobis(2-nitrobenzoic acid).

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(9 × 19 mm) upper and lower chambers were separated by a sheet (2.5 × 4 cm) of 1-mm dialysis tubing, which had been boiled twice for 30 min in 5% Na_2CO_3 and once for 30 min in 50 mM EDTA, pH 7.0. The tubing was stored at 4 °C in 1 mM EDTA, pH 7.0, and washed with distilled water before use. The inlet to the lower chamber was connected to a Mariotte flask reservoir, positioned to maintain a constant flow rate of 8 mL/min. The outlet of the bottom chamber was connected to a fraction collector. All the binding studies were performed with a pH 7.0 buffer containing 25 mM β-glycerol-P, 25 mM glycylglycine, 1 mM EDTA, and 1 mM DTT. After the lower chamber (2.8 mL) was filled with buffer and connected both to the reservoir containing the binding buffer and to the fraction collector, phosphofructokinase (0.5–2 mg/mL) was then added to the upper chamber. Fractions of 2 mL were then collected, and after the fifth fraction, [2,8-^{3}H]cAMP (10^6 cpm/mL) was added to the upper chamber to a final volume of 1.5 mL. The rate of dialysis of the ligand across the membrane equilibrated with the flow rate through the bottom chamber in approximately 2 min. Subsequently, every 3 min 10 μL of unlabeled cyclic AMP (0.75 nmol) was added while 2-mL fractions were continuously collected. Aliquots (1 mL) of each fraction were combined with 10 mL of Scinti-verse cocktail in plastic counting vials and counted in a scintillation counter. The rate of dialysis and the number of counts representing 100% free ligand were determined from a Scatchard-like plot of bound vs. bound/free ligand. The radioactivity, which dialyzed from the upper to the lower chamber, represented the amount of free ligand in the upper chamber. When the rate of dialysis equilibrated with the flow through the bottom chamber, the counts per minute per milliliter collected reflected the concentration of free ligand in the upper chamber. The amount of bound ligand was calculated by subtracting the amount of free ligand from the total ligand concentration. At the end of an experiment, a large excess of unlabeled ligand was added to displace all the bound radioactive ligand from phosphofructokinase. The counts per minute per milliliter value at this juncture represented 100% free ligand. A plot of log dialysis rate vs. log [cyclic AMP] was linear and had a slope equal to 1, which meant that during the course of an experiment the dilution in the specific activity of the radioactive ligand by the addition of unlabeled ligand was exactly compensated by the rate of dialysis. The presence of additional ligands did not affect the linearity of the relationship.

Thiol Titrations. Titrations of the phosphofructokinase sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) were performed as previously described by Kemp & Forest (1968). The reactions were carried out at 20 °C in a pH 7.0 buffer containing 25 mM β-glycerol-P, 25 mM glycylglycine, and 1 mM EDTA with approximately 0.2 mg/mL phosphofructokinase in a final volume of 3 mL. The reaction was initiated by the addition of various amounts of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma). A millimolar extinction coefficient of 13.6 (Ellman, 1959) at 412 nm for the thionitrobenzoic acid ion was used. The reaction was followed spectrophotometrically with an increase in absorbance at 412 nm with time.

Arylation Reactions. The reaction was carried out at room temperature in a total volume of 0.2 mL in a pH 7.0 buffer consisting of 25 mM β-glycerol-P, 25 mM glycylglycine, and 1 mM EDTA. Approximately 0.03 mg of phosphofructokinase was incubated for 5 min with the indicated concentration of nucleotide, prior to the addition of 10 μL of fluorodinitro-[^{14}C]benzene–ethanol solution (1.05×10^4 cpm, 2.38 nmol). The reaction was terminated after 1 min by applying a 50-μL aliquot of the reaction mixture onto a Whatman 3 MM filter (2 × 2 cm) and immersing the filter in cold 10% trichloroacetic acid (10 mL/filter). The filters (10 mL/filter) were subsequently washed twice with 5% trichloroacetic acid and twice with 100% ethanol at 4 °C for 10 min. After air-drying, the filters were placed in a plastic scintillation vial, to which 6 mL of toluene-based scintillation fluor was added, and counted in a scintillation counter. Under these conditions, the counting efficiency was found to be 55% for ^{14}C.

Modification of Phosphofructokinase with 8-Azido-cAMP. The photolysis of 8-azido-cAMP was performed in a reaction vessel consisting of two glass test tubes (12 × 75 mm) positioned adjacent to each other and 3 cm from a UVS-11 mineral light, which was positioned horizontally parallel to the two test tubes. The cover of the lamp was removed, and the two test tubes were aligned to ensure they were both exposed to equal intensities of light. Identical results were obtained for samples photolyzed under the same conditions but in different tubes. The photolysis was performed in 25 mM β-glycerol-P, 25 mM glycylglycine (pH 7.0 or 8.0), 1 mM EDTA, and 2.5 mM 2-mercaptoethanol. Phosphofructokinase and 8-azido-cAMP were added to the final concentrations indicated in the results and allowed to equilibrate in the dark for 5 min at room temperature before irradiation. The covalent incorporation of 8-azido[^{32}P]cAMP (ICN, Irvine, CA) into phosphofructokinase was determined following the procedure of Bollum (1968). Aliquots (50 μL) of the photolyzed solution were absorbed onto 2.3-cm Whatman 3 MM filter disks. The disks were washed (10 mL/disk) successively with 10% Cl_3CCOOH, twice with 5% Cl_3CCOOH, and twice with 100% ethanol. The ethanol was evaporated with a heat lamp, and each disk was counted in a scintillation counter.

Enzyme Assays. Assays to determine phosphofructokinase activity were performed at 30 °C either at pH 7.0 or at pH 8.2 with a recording spectrophotometer (Kemp, 1975). The pH 7.0 assay, which was used for measuring regulatory properties, contained in a 3-mL cuvette 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 0.15 M KCl, 1 mM DTT, 1 mM EDTA, 0.2 mM NADH, 1 mM fructose 6-phosphate, 0.6 unit/mL aldolase, 0.3 unit/mL glycerol-P dehydrogenase, and 0.3 unit/mL triose-P isomerase. The magnesium ion concentration was always maintained in 5 mM excess of the indicated nucleotide concentrations. The auxiliary enzymes, which were obtained from Sigma Chemical Co. (St. Louis, MO), were desalted either by passage through a Sephadex G-25 column (0.8 × 16 cm) previously equilibrated with 25 mM β-glycerol-P, 25 mM glycylglycine, and 1 mM EDTA, pH 7.0, at 4 °C or by dialyzing them against two changes of 100 volumes of the same buffer at 4 °C. After a 5-min preincubation at 30 °C, the phosphofructokinase reaction was initiated with the addition of fructose 6-phosphate. Phosphofructokinase was diluted with a pH 7.0 buffer containing 25 mM β-glycerol-P, 25 mM glycylglycine, 1 mM EDTA, 1 mM DTT, and 0.1 mM ATP. The assay at pH 8.2 for total activity contained 25 mM β-glycerol-P, 25 mM glycylglycine, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.2 mM NADH, 3 mM $(NH_4)_2SO_4$, 1 mM ATP, 1 mM fructose 6-phosphate, 0.6 unit/mL aldolase, 0.3 unit/mL glycerol-P dehydrogenase, and 0.3 unit/mL triose-P isomerase. Prior to the addition of the enzyme to the assay, phosphofructokinase (PFK) was diluted with 25 mM β-glycerol-P, 25 mM glycylglycine (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.1 mM ATP.

Other Methods. The cyclic AMP derivatives, *N*-6-(2-aminoethyl)-cAMP, *N*-6-(4-aminobutyl)-cAMP, and *N*-6-(6-aminohexyl)-cAMP, were synthesized on a microscale, by a modification of the method of Dills et al. (1975). A 5-mg (13-mmol) aliquot of 6-chloro-cAMP was added to a 1-mL solution of either 50 mg of ethylenediamine dichlorochloride, putrescine, or 1,6-hexanediamine. The solutions were refluxed for 6 h and then cooled. The products were isolated on a Dowex 1-X2 column (0.8 × 20 cm, acetate form). The column was washed sequentially with 8 mL of distilled water, 8 mL of 10 mM NH_4Cl, and 8 mL of distilled water. The derivatives were eluted from the column with a 0–0.5 N acetic acid linear gradient; the total gradient volume was 40 mL. Fractions containing both ninhydrin-positive and UV-absorbing (256 nm) material were pooled and lyophilized. The dried products were dissolved in 0.5 mL of distilled water and adjusted to pH 7.0 with NaOH. The nucleotide concentrations were determined by measuring the amount of inorganic phosphate (Ames, 1965) liberated after ashing the organic phosphate compounds. KH_2PO_4 and cyclic AMP were used as standards to ensure the completeness of the ashing.

8-Amino-cAMP was produced by incubation of 8-azido-cAMP at room temperature in 25 mM glycylglycine, 25 mM β-glycerol-P, 1 mM EDTA, and 1 mM DTT. Thin-layer cellulose chromatography indicated a rapid and virtually stoichiometric conversion of 8-azido-cAMP to the 8-amino product in the presence of DTT. [2,8-^{3}H]cAMP (Amersham, Arlington Heights, IL) was purified on Whatman MN 300 cellulose TLC plates. The plate was developed for 3 h by ascending chromatography with an equilibrated mixture of *n*-butanol, acetone, acetic acid, 28–30% ammonia, and water (35:25:15:2.5:12.5 v/v). After air-drying overnight, the plate was scraped, and [^{3}H]cAMP was determined by using a scintillation counter. The radioactivity that ran parallel with the UV-absorbing standard of cyclic AMP was stored at −15 °C. On the day of an experiment, the powder was suspended in the binding buffer for several minutes and subsequently centrifuged for 2 min in an Eppendorf centrifuge. Protein concentration was determined by the Coomassie dye assay as described by Bradford (1976) with ATP-free phosphofructokinase as a standard or spectrophotometrically at 290 nm in 0.1 N NaOH by using an absorption coefficient of 1.09 mL mg^{-1} cm^{-1} or at 280 nm, pH 7.0, with an absorption coefficient of 1.01 mL mg^{-1} cm^{-1} (Paetkau & Lardy, 1967).

Results

Dinucleotide Binding to Phosphofructokinase. A study of dinucleotide binding to phosphofructokinase was based on two previous observations: (1) phosphofructokinase binds specifically to blue dextran, a diagnostic probe for dinucleotide folds (Bohme et al., 1972), and, (2) like several NADH-dependent dehydrogenases, phosphofructokinase is modified by the adenine derivative 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Pettigrew & Frieden, 1978). Modification of the adenine nucleotide site with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine suggested that NADH might bind at the same site as cyclic AMP.

The interaction of dinucleotides with phosphofructokinase was studied indirectly on the basis of the ability of the dinucleotides to displace cyclic AMP from the enzyme. Han et al. (1977) reported that commercial preparations of NADP were contaminated with trace amounts of AMP, enough to inhibit the activity of muscle fructose diphosphatase when assayed with 0.2 mM $NADP^+$. The adenine nucleotide binding site of PFK does not recognize IMP (Kemp & Krebs, 1967); therefore, all the mono- and dinucleotides that are not substrates for adenylic deaminase were treated with adenylic deaminase to convert any contaminating AMP to IMP. The nucleotides (10 mM) were incubated with 2 units of adenylic deaminase at 30 °C for 15 min in a pH 7.0 buffer consisting of 25 mM β-glycerol-P, 25 mM glycylglycine, and 1 mM EDTA. A 10 mM solution of AMP was treated with adenylic deaminase under the same conditions to ensure that the reaction was complete. AMP was completely converted to IMP within 15 min. The adenylic deaminase was removed by ultrafiltration under nitrogen, using an Amicon ultrafiltration cell fitted with a PM-10 filter.

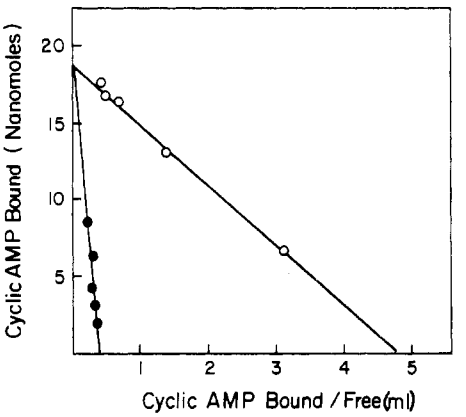

FIGURE 1: Scatchard plot for phosphofructokinase binding of cyclic AMP in the presence and absence of ADPR. Buffer (pH 7.0) and other conditions are described under Materials and Methods. (○) No addition; (●) plus 0.1 mM ADPR.

Table I: Inhibition of Cyclic AMP Binding by Dinucleotides[a]

competing ligand	K_i (μM)
ADPR	5.1
NADH	17.1
NAD^+	57
NADPH	41
$NADP^+$	48
NMN	102

[a] Binding of cyclic AMP in the presence and absence of other ligands was performed as described under Materials and Methods. The K_i values for the dinucleotides and the dinucleotide fragments were calculated from the change in slope of the Scatchard plot equal to $1 + [I]/K_i$, where [I] is the concentration of the inhibitor. The K_d value for cyclic AMP, which was determined directly from the slope of the Scatchard plot, was 1.3 μM.

When measured by the fast-flow equilibrium dialysis technique, the dissociation constant for the cyclic AMP–enzyme complex was found to be 1.3 μM at pH 7.0, which is similar to the value determined by Kemp & Krebs (1967) by using the technique of Hummel & Dreyer (1962). Figure 1 describes the binding of cyclic AMP in the presence and absence of ADPR. The K_i for ADPR, which would be equivalent to its dissociation constant, was calculated from the change in slope of the Scatchard plot, with the slope change equal to $1 + [I]/K_i$. Similar studies were performed with all of the nicotinamide adenine dinucleotides, and all behaved as purely competitive inhibitors of cyclic AMP binding. These results are described in Table I. The data are compatible with the suggestion that the dinucleotides and dinucleotide fragments bind to the adenine nucleotide site with the affinities indicated in the table. ADPR was bound the tightest with an affinity approximately one-fourth that of cyclic AMP. Of the dinucleotides, NADH bound the tightest and about 2–3 times better than NAD, NADP, and NADPH. The oxidized and reduced forms of the nicotinamide dinucleotide had different affinities, but the dissociation constants for the oxidized and

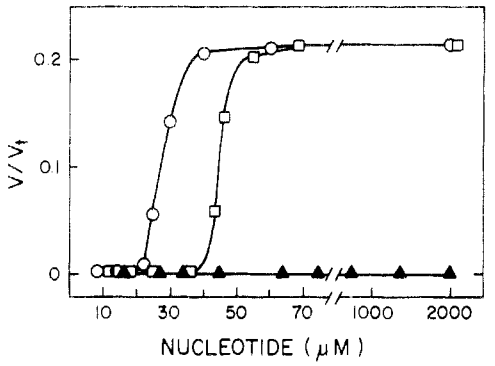

FIGURE 2: Activation of ATP-inhibited phosphofructokinase by cyclic AMP and ADPR. V_t is maximal activity as determined at pH 8.2. Assays were performed at pH 7.0 with 1 mM fructose-6-P and 3 mM ATP, and other conditions are described under Materials and Methods. Under these conditions, the enzyme 2% of the maximal activity at pH 7.0. (○) Cyclic AMP as added nucleotide; (□) cyclic AMP as added nucleotide plus 0.67 mM ADPR; (▲) ADPR as added nucleotide.

reduced nicotinamide adenine dinucleotide phosphates were equivalent. NMN, which lacks the adenine ring, bound the least tightly. The affinity of the dinucleotides for the adenine nucleotide binding site appeared to be enhanced by the adenine ring and reduced by the nicotinamide moiety and the 2′-hydroxy phosphate of the nicotinamide adenine dinucleotide phosphates.

Brock (1969) reported that NADH inhibited phosphofructokinase at pH 7.0, and this observation has made its way into a number of recent textbooks. Newsholme et al. (1970) later demonstrated, however, that the NADH inhibition was really due to inhibition of the auxiliary enzymes used to assay phosphofructokinase. He showed that inhibition of phosphofructokinase at high NADH concentrations was abolished by increasing the auxiliary enzymes or using a nonspectrophotometric pH stat assay. The spectrophotometric phosphofructokinase assay used in the present study initially contains 0.2 mM NADH. Therefore, any observable kinetic effect of the dinucleotides would be that seen over and above the effect of 0.2 mM NADH on the basal activity of phosphofructokinase. Because ADPR bound tightly to the adenine nucleotide site and does not absorb light at 340 nm, ADPR was employed as a representative dinucleotide for studying the possible regulatory behavior of dinucleotides. Figure 2 compares the kinetic effect of cyclic AMP and ADPR on the activity of ATP-inhibited phosphofructokinase. In the experiment, sufficient ATP was added to produce 98% inhibition of the maximal activity at pH 7.0, and the deinhibiting action of cyclic AMP and ADPR was determined. Because of potential ADPR inhibition of the auxiliary enzymes, these three enzymes were tripled in assays with ADPR. Under these conditions, the coupling enzymes were not limiting as indicated by assays at low, noninhibitory ATP concentrations. Contrary to what was anticipated, ADPR did not activate phosphofructokinase. As seen in Figure 2, in the presence of 3 mM ATP, phosphofructokinase was half-maximally activated by 27 μM cyclic AMP, whereas ADPR was not effective even at 2 mM concentrations. Because the binding studies demonstrated that the dinucleotides competitively bind to the adenine nucleotide site, the ADPR should at least competitively inhibit the cAMP-dependent activation of phosphofructokinase. This was shown by directly reversing the activation at an inhibitory concentration (3 mM) of ATP and a deinhibiting concentration of cyclic AMP (33 μM); 50% inhibition of the enzyme could be achieved with 0.25 mM ADPR. The apparent ADPR inhibition of cyclic AMP activation is readily

Table II: Thiol Reactivity in the Presence of Added Ligands[a]

additions	mol of SH reacted/protomer in 2 min	in 12 min
none	2.8	3.8
cyclic AMP	1.7	2.5
ADPR	1.3	2.5
NADH	1.4	2.2
NAD	1.6	2.9
NADPH	1.7	2.8
NADP	1.4	2.6
NMN	1.8	3.1

[a] Phosphofructokinase (0.21 mg/mL) was reacted at 20 °C with 33 μM DTNB in a pH 7.0 buffer consisting of 25 mM β-glycerol-P, 25 mM glycylglycine, and 1 mM EDTA. All other additions were present at 1 mM. Reactions were followed at 412 nm as described under Materials and Methods.

reversed by increasing the concentration of cyclic AMP (Figure 2). In the presence of 3 mM ATP, 0.67 mM ADPR increased the concentration of cyclic AMP necessary for half-maximal activation from 27 to 42 μM. ADPR appeared to compete for adenine nucleotide binding and to shift the activity curve for cyclic AMP activation to higher cyclic AMP concentrations, but it did not itself reverse ATP inhibition.

ADPR and Conformational Changes in Phosphofructokinase. The finding of apparent competition of dinucleotides with cyclic AMP binding but the absence of a similar mode of kinetic action suggested that the binding of ADPR may not induce the conformational change seen with the activators. Perhaps the dinucleotides which have a more extended conformation than cyclic AMP elicit a different conformational change than that seen with cyclic AMP. In earlier studies, this laboratory has employed thiol reactivity as a monitor of the conformational state of the enzyme (Kemp & Forest, 1968; Mathias & Kemp, 1972). Phosphofructokinase has four sulfhydryl groups that are rapidly titrated with DTNB at pH 7.0 (Kemp & Forest, 1968). Of the four, the single most reactive thiol groups is referred to as the class I thiol. The adenine nucleotides and fructose 6-phosphate shift phosphofructokinase into a conformation where two of the four reactive thiols were protected from reacting with DTNB. These two thiol groups have been called class II thiols (Kemp & Forest, 1968). Table II compares the protective effect of 1 mM cyclic AMP with 1 mM NAD, 1 mM NADH, 1 mM NADP, 1 mM NADPH, 1 mM NMN, and 1 mM ADPR on thiol reactivity at two time points in the course of the reaction of DTNB with phosphofructokinase thiol groups. In agreement with earlier studies, thiol protection by cyclic AMP is observed. ADPR and all of the dinucleotides were capable of protecting the thiol groups. NMN appeared to have the weakest protective action. Aside from the slight differences in the number of moles of SH titrated per mole of protomer, second-order reaction plots for the mononucleotides and the dinucleotides were the same. These results thus show that with respect to the class II thiol groups dinucleotides induce the same conformational change as do the adenine mononucleotides. Of significance to the active–inactive conformational shift is the class I thiol group (Kemp & Forest, 1968), whose reactivity is inhibited by the interaction of MgATP with the inhibitory site and by the other inhibitors, citrate and phosphoglycerate (Kemp, 1969; Mathias & Kemp, 1972). The reactivity of the class I thiol is increased by the activators fructose-6-P, fructose-1,6-bisP, cyclic AMP, AMP, and ADP (Mathias & Kemp, 1972). On the basis of these data, it was predicted that ADPR should be unable to promote the reactivity of the class I thiol group in view of its inability to reverse the inhibition of phosphofructokinase. This

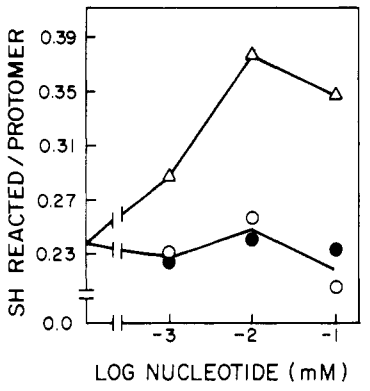

FIGURE 3: Effect of cyclic AMP and ADPR on the arylation of phosphofructokinase. Arylation of phosphofructokinase at 0.15 mg/mL with fluorodinitrobenzene for 1 min at 23 °C was performed as described under Materials and Methods. (△) Cyclic AMP as added nucleotide; (○) ADPR as added nucleotide; (●) cyclic AMP as added nucleotide plus 0.1 mM ADPR.

was examined by measuring the rate of the reaction of the class I thiol group with fluorodinitrobenzene in the absence and presence of cyclic AMP or ADPR. Fluorodinitrobenzene reactivity was followed because the reaction is slower, and increased reaction rates are more easily determined. Figure 3 shows these results. As predicted, ADPR over a wide range of concentrations was unable to increase the rate of reaction of the class I thiol group whereas such an increase was easily observed with cyclic AMP, a nucleotide whose affinity for the adenine nucleotide site is similar to that of ADPR. Of particular interest was the observation that ADPR could reverse the effect of cyclic AMP on thiol reactivity by preventing the binding of cyclic AMP at the adenine nucleotide site. Thus, ADPR readily binds to the adenine nucleotide site but does not induce the same conformational change as does AMP or cyclic AMP.

Other Properties of the AMP (cAMP) Binding Site. An attempt was made to modify the adenine nucleotide site covalently by photoaffinity labeling with 8-azido-cAMP. Although up to 0.3 mol/mol of 8-azido[^{32}P]cAMP could be incorporated, the photolabeled product did not possess the properties expected of an enzyme labeled at the cyclic AMP binding site. A decrease in activity was seen, and the partially modified enzyme was fully sensitive to deinhibition by cyclic AMP. Furthermore, the photomodification was poorly protected by cyclic AMP, particularly at pH 8.0. These results suggested that labeling occurred at or near the catalytic site, a conclusion also reached in a recent paper by Lascu et al. (1979), who employed 8-azido-AMP as a photolabel for phosphofructokinase. In any event, it appeared that substitution at the 8 position of adenine may reduce or prevent binding at the nucleotide site.

Binding of 8-azido-cAMP, in the dark, was examined by competition for cyclic AMP binding in the fast-flow method previously described. Because dithiols will rapidly react with aryl azides in the dark to produce arylamines (Staros et al., 1978), the monothiol 2-mercaptoethanol was substituted for DTT. The relative rate for the dark reaction of azidoadenosine in a 2-mercaptoethanol solution is negligible compared to that for an equimolar solution of DTT. On the basis of a competitive binding assay, 8-azido-cAMP bound about 100-fold less tightly than cyclic AMP to the adenine nucleotide site. At pH 7.0, the apparent dissociation constant for 8-azido-cAMP was 110 μM (Table III). Because ADPR binds at the adenine nucleotide site but does not activate phosphofructokinase (Figure 2), it was of interest to determine what effect the modification of the 8-carbon of cyclic AMP had on

Table III: Binding and Kinetic Effects of Cyclic AMP Derivatives[a]

ligand	app K_d (μM)	app K_a (μM)
cAMP	1.3	50
8-azido-cAMP	110	2900
8-amino-cAMP	9.2	ND
N-6-(4-aminobutyl)-cAMP	ND	>3000
N-6-(6-aminohexyl)-cAMP	>1000	>3000

[a] The app K_d was calculated from competitive binding assays as described under Materials and Methods. Apparent K_a was determined by measuring the concentration required to give half-maximal stimulation in the pH 7.0 assay containing 3 mM ATP, 1 mM fructose-6-P, and other additions as given under Materials and Methods. In the absence of added ligands, the velocity was 2% of maximal velocity under these conditions. ND = not determined.

the derivatives's ability to deinhibit phosphofructokinase in the presence of inhibitory ATP. As indicated in Table III, 8-azido-cAMP mimicked the activating effect of cyclic AMP, but at a concentration more than 50-fold higher than that required by cyclic AMP to produce the same effect. Modification of the 8 position appeared to only decrease the affinity of the cyclic nucleotide for phosphofructokinase. On the other hand, a less bulky substituent at the 8-carbon has a much less pronounced effect on the affinity of cyclic AMP. 8-Amino-cAMP could compete with cyclic AMP binding much more effectively than the azido derivative. The calculated K_d for the 8-amino derivative was approximately only 7 times higher than the K_d for cyclic AMP.

Substitution at Position N-6. In the course of attempting to develop a cyclic AMP affinity chromatography method for the purification of phosphofructokinase, the interaction of *N*-6-aminoalkyl derivatives of cyclic AMP with the enzyme was examined. Initially, a commercial preparation of *N*-6-(6-aminohexyl)-cAMP was coupled to CNBr-activated Sepharose 4B as described by Lindberg & Mosbach (1975). Phosphofructokinase absorbed onto the cyclic AMP–Sepharose column at room temperature. Subsequent additions of 1, 5, 10, and 50 mM concentrations of cyclic AMP were not able to remove the immobilized enzyme. This suggested that instead of a specific affinity of phosphofructokinase for the bound cyclic AMP hydrophobic and/or ionic interactions were involved in retaining the protein on the column. Increasing the ionic strength of the eluting buffer, by using 0.1 M potassium phosphate (pH 7.0), 1 mM DTT, 1 mM EDTA, and 1 mM fructose diphosphate, eluted greater than 98% of the enzyme initially applied to the column, in a single, sharp, and symmetrical peak. Placing the column in the cold (4 °C) or equilibrating the column with a higher ionic strength buffer, prior to adding the protein to the column, prevented the adsorption or enzyme by cyclic AMP–Sepharose. Furthermore, phosphofructokinase adsorbed to the affinity column in the presence of 1 mM cyclic AMP. All of these results suggested a nonspecific hydrophobic interaction as the basis for the enzyme binding to the column. Dills et al. (1975) overcame a similar problem of hydrophobic affinity while purifying the regulatory subunit of protein kinase by employing cyclic AMP derivatives with shorter coupling arms on the N-6 position.

With this in mind, *N*-6-(2-aminoethyl)-cAMP, *N*-6-(4-aminobutyl)-cAMP, and *N*-6-(6-aminohexyl)-cAMP were synthesized, and they were tested for their ability to activate ATP-inhibited phosphofructokinase at pH 7.0. The results are summarized in Table III. A 50-μM aliquot of cyclic AMP activated phosphofructokinase that had been inhibited by 3 mM ATP, but the *N*-6-(6-aminohexyl)-cAMP and *N*-6-(4-aminobutyl)-cAMP could not deinhibit the enzyme even at

concentrations as high as 0.28 and 1 mM, respectively. When added with 50 μM cyclic AMP, the aminoalkyl cyclic AMPs did not affect the activation of phosphofructokinase by cyclic AMP. These results suggest that the aminoalkyl derivatives either had a very low affinity for the adenine nucleotide site or did not bind at all. The dissociation constants for *N*-6-aminoalkyl cAMPs were also determined as K_i values from competitive inhibition of cyclic AMP binding. These results are summarized in Table III. The K_i was greater than the sensitivity of the method. Similar results were obtained with *N*-6-(2-aminobutyl)-cAMP (not shown in Table III). It is apparent that substitution of the N-6 position of the purine ring by an alkylamine drastically interferes with the binding to the adenine nucleotide site on phosphofructokinase. It is also obvious that such derivatives are inappropriate for use as affinity chromatography reagents.

Discussion

Phosphofructokinase possesses three distinct ATP binding sites per protomer: the catalytic site, the ATP inhibitory site, and the adenine nucleotide activating site (Kemp & Krebs, 1967). These sites differed from each other with respect to their ligand specificities and biological actions. The results of this study provide further insight into the stereochemical requirements of the adenine nucleotide site and evidence of an overlapping dinucleotide fold.

Cyclic AMP and the dinucleotides, NADH, NAD, NADPH, and NADP, bind competitively to phosphofructokinase. The data are compatible with the suggestion that dinucleotides are capable of interacting with the adenine nucleotide activating site of the enzyme. The dissociation constants for dinucleotides were calculated as K_i values from competitive inhibition of cyclic AMP binding (K_D = 1.3 μM). The binding of the dinucleotide fragments, ADPR (K_D = 5.1 μM) and NMN (K_D = 102 μM), to phosphofructokinase suggests the congruency of the adenine nucleotide site with a larger dinucleotide binding domain. The weak binding of NMN and the very tight binding of ADPR emphasize the predominant contribution of the adenine moiety to the affinity of dinucleotides for phosphofructokinase. In contrast to NAD-dependent dehydrogenases, phosphofructokinase had a greater affinity for ADPR than for either the oxidized or the reduced nicotinamide adenine dinucleotide.

While adenine nucleotides (AMP, ADP, and cyclic AMP) activated ATP-inhibited phosphofructokinase, ADPR, taken as representative of dinucleotides, did not affect the activity of the enzyme. ADPR, however, could displace to higher concentrations the concentration of cyclic AMP required to produce an equivalent degree of activation to that observed in the absence of ADPR. The inability of ADPR to activate the enzyme could be predicted on the basis of conformational studies that employed the reactivity of the class I thiol group as an indication of the active–inactive transition. On the other hand, ADPR and the other dinucleotides mimicked the protective effect of a cAMP-induced conformational change on the two class II thiols of phosphofructokinase. It has been suggested previously (Kemp et al., 1976) that the protection of these thiols by either adenine nucleotides or fructose-6-P only reflects a local conformational change within the protein and not an overall shift in the allosteric equilibrium of phosphofructokinase. Fructose diphosphate, which is also an activator, has no effect on the class II thiol reactivity (Kemp & Forest, 1968) but does increase the reactivity of the class I thiol (Mathias & Kemp, 1972).

The nucleotide analogue's ability to bind to an allosteric site, but failure to exert a concomitant protein conformational change, is not unique to ADPR and phosphofructokinase. Salicylate, an adenine nucleotide analogue, competitively bound to the AMP allosteric inhibitory site of fructose diphosphatase (Marcus, 1976) but did not induce the anticipated conformational change of fructose diphosphatase to an inhibited protein state. The catalytic activity of fructose diphosphatase was not inhibited by salicylate; however, salicylate did relieve the allosteric AMP inhibition.

The observed low affinity of azido-cAMP for the adenine nucleotide site of phosphofructokinase may reflect the different glycosidic conformations (orientation of the adenine ring about the ribose C′-1-adenine–N-9 bond with respect to the ribose ring) that cAMP and 8-azido-cAMP possess. Cyclic AMP exists primarily in the anti conformation, where the C-8 group of the purine ring lies over the ribose ring. Substitution at the 8-carbon position of the adenine ring with a bulky group (such as Br) causes a steric hinderance with both the sugar and the ribose phosphate group. This destabilizes the anti conformation and leads to a 180° rotation about the glycosidic bond to the more favorable syn conformation (Miles et al., 1979). If phosphofructokinase favors the anti conformation, the thermodynamic stability of the syn conformation for 8-azido-cAMP (Lascu et al., 1979) would explain the poor affinity of azido-cAMP for the adenine nucleotide site of phosphofructokinase. It should be noted that the less bulky 8-amino-cAMP binds more tightly.

Phosphofructokinase does not specifically absorb to *N*-6-(6-aminohexyl)-cAMP–Sepharose, but only interacts electrostatically with the affinity gel as shown by the fact that the enzyme binds to the affinity column even in the presence of free cyclic AMP. Increasing the ionic strength of the eluate abolishes the nonspecific affinity of the enzyme for cyclic AMP–Sepharose. The inability of phosphofructokinase to specifically absorb to cyclic AMP–Sepharose is in agreement with the failure of N-6-alkylated derivatives to bind to the adenine nucleotide site of phosphofructokinase. Possibly, the additional bulk at the N-6 position of the cyclic AMP derivatives sterically hinders the binding of the aminoalkyl cyclic AMPs. These results provide further contrast of the specificity of the adenine nucleotide activation site with the nucleoside triphosphate catalytic site. As opposed to the present observations, the catalytic site of phosphofructokinase apparently interacts readily with ATP substituted at the N-6 position (Ramadoss et al., 1976).

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Photosuicide Inactivation of Acetylcholinesterase by Nitrosamine Derivatives†

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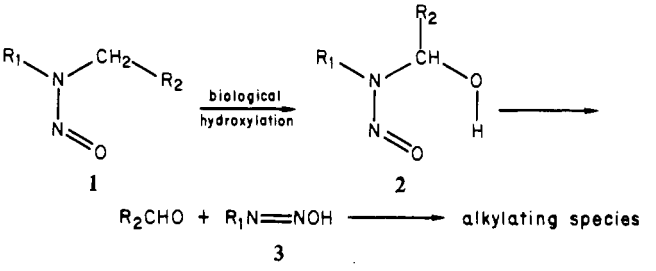
ABSTRACT: Methyl(acetoxymethyl)nitrosamine and methyl(butyroxymethyl)nitrosamine are respectively substrate (K_M = 10^{-2} M) and competitive inhibitor (K_i = 2 × 10^{-3} M) of electric eel acetylcholinesterase (EC 3.1.1.7). Irradiation of an incubation mixture of this enzyme with either nitrosamine leads to an irreversible loss of enzyme activity. The inactivation rates are dependent on photolysis wavelength, light intensity, and inhibitor concentration. Experiments where acetylcholinesterase was radioactively labeled by [^{14}C]-methyl(acetoxymethyl)nitrosamine show that the incorporation of 1 mol of radioactive label per active site is sufficient to cause complete enzyme inactivation irrespective of the reaction conditions used. Methyl(acetoxymethyl)nitrosamine shows no affinity for horse serum butyrylcholinesterase (EC 3.1.1.8) while methyl(butyroxymethyl)nitrosamine is a competitive inhibitor (K_i = 2 × 10^{-3} M), but no irreversible inhibition is induced by the action of light. We propose that a suicide type of inhibition [Bloch, K. (1969) *Acc. Chem. Res. 2*, 193–198] is responsible for the inactivation of acetylcholinesterase, based on photoactivation of nitrosamines only when associated with an acidic hydrogen of the active site.

Nitrosamines are among the most powerful carcinogenic chemicals known. They are easily converted after in vivo α-hydroxylation to alkylating species (Skipper et al., 1977), which are responsible for their carcinogenicity (Scheme I) [for a recent review, see Ingelman-Sundberg (1980)].

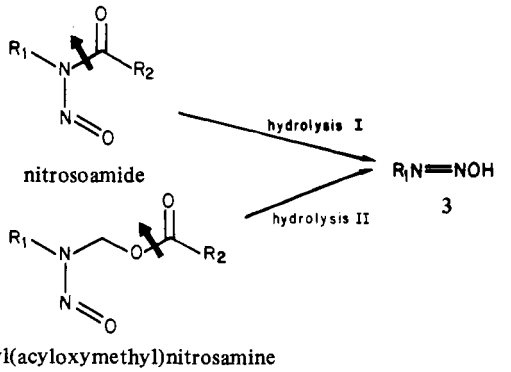
Diazoïc acid **3**, a precursor of carbonium ions, is the key intermediate in this transformation. One can use the alkylating power of those ions to inactivate enzymes. In addition, if precursor **3** of these carbonium ions is produced and decomposed at the active site of the enzyme, the conditions necessary for suicide inhibition of the enzyme have been fulfilled (Bloch, 1969; Rando, 1974, 1975; Abeles, 1976). Such a principle can be readily applied to the suicide inhibition of esterases. As a matter of fact, there are several ways to generate diazoïc acid **3** by using hydrolytic reactions (Scheme II).

Successful catalytic inhibition of chymotrypsin has been described by White et al. (1975, 1977a,b, 1978) on nitrosoamide hydrolysis. The use of nitrosamine derivatives (hydrolysis II) was of interest because this starting material is much more stable at physiological pH than the corresponding nitrosoamide and might allow studies of complex systems or in vivo experiments.

Scheme I



Scheme II



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0006-2960/81/0420-2251$01.25/0