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Synthesis of an Adenosine 5′-Monophosphate Analog and Its Use for the Affinity Labeling of the Effector Binding Site of Rabbit Skeletal Muscle Phosphorylase *b*†

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ABSTRACT: The synthesis of 6-(purine 5′-ribonucleotide)-5-(2-nitrobenzoic acid) thioether provided a reagent that will form stable thioether bonds between the 6 position of the purine moiety and aliphatic sulfhydryls. 2-Nitro-4-mercaptobenzoic acid is eliminated during this reaction. The nucleotide reagent, labeled with ^{32}P, was used to activate phosphorylase *b* from rabbit muscle. The activation showed a stoichiometric relation to the amount of nucleotide incorporated into the enzyme. The nucleotide was covalently linked to the protein. We propose that the nucleotide became bound at or near the 5′-AMP binding site of the enzyme.

Affinity labeling of enzymes by substrate analogs which form covalent bonds with amino acid side chains of the protein is an useful and well-established technique in enzymology. Although a large number of nucleotide analogs have been synthesized (Follmann *et al.*, 1967; Holy, 1970) for the purpose of studying enzyme mechanisms, relatively few of them were designed to form covalent bonds with protein side chains (Cuatrecasas, 1970; F. Eckstein, personal communication).

Experiments on side-chain modifications at or near the 5′-AMP binding site in phosphorylase *b* (H. Fasold *et al.*, 1971, unpublished data) suggested the need for a protein-reactive 5′-AMP analog. Previous studies had already suggested the participation of a sulfhydryl group in 5′-AMP binding (Damjanovich and Kleppe, 1967; Batell *et al.*, 1968; Gold, 1968; Kastenschmidt *et al.*, 1968). Therefore, we tried to synthe-

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size a mixed disulfide from the 6-SH-AMP analog and 2-nitro-4-mercaptobenzoic acid. In this reaction, under the experimental conditions used by us, a thioether linkage was formed and the sulfur of the nucleotide was eliminated. In the presence of aliphatic thiol compounds the thioether was cleaved and a new and more stable thioether linkage with the nucleotide moiety formed, while the 2-nitro-4-mercaptobenzoic acid was released. When muscle phosphorylase *b* was treated with the thioether reagent, the enzyme was partially activated by the stoichiometrically and covalently bound nucleotide.

Experimental Section

Material and Methods. 6-Mercaptopurine ribonucleoside was obtained from Waldhof Chemie, Mannheim. For protection, the isopropylidene group was introduced by the procedure of Hampton and Magrath (1957). ^{35}S-Labeled 5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent,[1] was purchased from EGA Chemie, Weinheim. ^{35}S-Labeled Ellman's reagent was synthesized from 2-nitro-5-chlorobenzoic acid. 2-Nitro-5-chlorobenoic acid (2 g) was dissolved in 50 ml of water and neutralized to pH 7.2 with 5 N NaOH. To unlabeled sodium sulfide (2.45 g) was added 2.5 mCi of radioactive sodium sulfide which had a specific activity of 5 mCi/μmole, and dissolved in 30 ml of water. Both solutions were combined, and the mixture was stirred for 2 hr at 50°. A solution of iodine (4 g in 100 ml of 5% KI in water) was added in small portions to the reaction mixture, until the orange color of the thiophenolate anion disappeared and the faint yellow color of the disulfide appeared. Upon acidification, the substance precipitated. The oily crystals were recrystallized from acetic acid. They had a specific activity of 1.1 μCi/mg.

Carboxymethylation of 2′,3′-*O*-isopropylidene-6-mercaptopurine ribonucleoside was brought about by slowly adding a neutralized solution of 2 g of iodoacetic acid in 5 ml of water to a solution of 1 g of the ribonucleoside in 20 ml of a 0.01 M $NaHCO_3$ solution. The pH was kept above 8.0 by addition of 0.5 N NaOH, when necessary. Upon acidification to pH 3.0, the carboxymethylated product precipitated as fine white crystals. It could be purified from a solution at pH 8 by reprecipitation at pH 3. The compound gave a single spot on paper chromatography in isopropyl–alcohol 1 N ammonium acetate (pH 5.5), or on paper electrophoresis at pH 6.5 in 0.01 N sodium acetate buffer. It showed a characteristic uv absorbancy spectrum with extinction maxima at 284 and 219 mμ. The molar extinction coefficient at 284 mμ was 17,800; mp 273°. *Anal.* Calcd: C, 47.11; H, 4.74; N, 14.65; S, 8.38. Found: C, 46.66; H, 4.74; N, 14.61; S, 8.20.

Phosphorylase *b* was prepared from rabbit muscle according to Fischer and Krebs (1958). Enzymatic activity was determined by measuring the phosphorylase reaction in the direction of glycogen synthesis essentially as described by Cori *et al.* (1943). The inorganic phosphate released from glucose-1-P was determined in an AutoAnalyzer system (Technicon). In the aliquots of the incubation mixture, the enzyme was inactivated by addition of one-tenth of their volume of 60% v/v $HClO_4$. From the time course of phosphate release initial velocities were determined, taking the approach to equilibrium into consideration. For this purpose the first-order rate constants were determined as described by Cori *et al.* (1943). The incubation media at 30° contained 50 mM sodium glycerophosphate–HCl (pH 6.8), 0.5 mM EDTA, 0.5% glycogen pretreated to remove traces of 5′-AMP according to Helmreich and Cori (1964), 40 mM glucose-1-P, 20 μg/ml of native enzyme in the presence of 5′-AMP, 50–70 μg/ml of analog-treated enzyme or native enzyme in the absence of 5′-AMP, and, in control runs, 1.5 mM 5′-AMP. The enzyme preparations used had a specific activity of 75–80 μmoles of phosphate/min per mg of enzyme at 30° under the above assay conditions. Activity measurements were made in 1 day with an enzyme solution prepared the same day from the crystal suspension.

5′-AMP was removed by filtration of the enzyme solutions through Sephadex G-25 or passing it over charcoal columns. Removal of the effector was determined by measuring the absorbancies of phosphorylase *b* solutions at 280 and 260 mμ (Kastenschmidt *et al.*, 1968). Furthermore, the activity of the enzyme resolved from 5′-AMP was determined before and after addition of 5′-AMP. The concentration of the 5′-AMP-free enzyme was determined by the method of Lowry *et al.* (1951), with phosphorylase *b* thoroughly dialyzed, lyophilized, and dried, as standard. All other protein determinations were carried out with the biuret method (Weichselbaum, 1946). An absorbancy coefficient at 280 mμ of 13.0/1% per cm was used.

Radioactivity was measured in a Packard Tri-Carb scintillation counter, Model 2704, with 3% gain and a discriminator setting at 50–700 for ^{32}P, 7% gain and a discriminator setting at 50–700 for ^{35}S. Protein samples were dissolved in 1 ml of Digestin (E. Merck Co., Darmstadt) prior to the addition of the scintillator fluid (4% Scintimix, E. Merck Co., Darmstadt, in toluene). The decay of radioactivity of the ^{32}P-labeled material was determined by counting [^{32}P]$POCl_3$ at different times during the experiments. All experiments with ^{32}P were carried out within 22 days after arrival of the [^{32}P]-$POCl_3$ used in the preparations.

For peptic fingerprints, protein samples were adjusted to pH 2.8 with HCl and thoroughly dialyzed against 0.001 M HCl in the cold room. The pH was brought to 1.9 with HCl, and 0.05% (w/w of protein) bovine pepsin was added. Twelve hours later the pH was readjusted to 1.9, and the same amount of pepsin was added. The peptide mixture was lyophilized after another 12 hr, and subjected to high-voltage electrophoresis. A total of 100 V/cm was applied and pyridine–acetate buffer (pH 6.5) was used. Chromatography was carried out in 1-butanol–pyridine–acetic–acid–water (30:20:6:24, v/v). No peptide or radioactive material remained at the origin. For autoradiography, the fingerprints were placed between two sheets of X-ray paper, which were pressed between 2-mm steel plates. Exposure time was 14 days.

$s_{20,w}$ values were determined in a Beckman Model E ultracentrifuge equipped with schlieren optics. The speed was 56,000 rpm. Bar angle was 75°. A valve-type synthetic boundary cell was used. The solutions contained 50 mM sodium glycerophosphate–HCl buffer (pH 6.8), 1.5 M 5′-AMP, and 7 mg/ml of enzyme at 20°.

[6-^{35}S]Mercaptopurine was a product of the Amersham Radiochemical Centre, England. It had a specific activity of 13 μCi/μmole. X-Ray paper for autoradiography was a gift from the Agfa-Gevaert Co., Leverkusen. [^{32}P]$POCl_3$, with a specific activity of 32 μCi/μmole, was a product of the Amersham Radiochemical Centre, England. Bovine pepsin was purchased from Serva, Heidelberg. Charcoal was obtained from E. Merck Co., Darmstadt. It was washed prior to use for chromatography with two volumes of 6 N HCl at 60°, two volumes of water, and two volumes of 5% NaCN solution. Finally the charcoal was washed ten times or more with five volumes of water (K. Kirschner, private communication).

[1] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

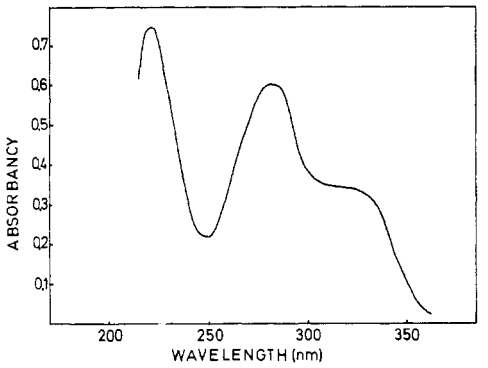
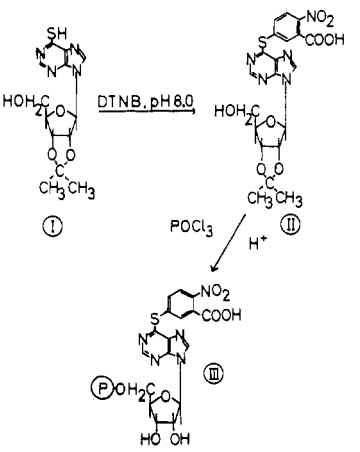


FIGURE 1: Uv absorbancy spectrum of II.

SCHEME I



Results

Synthesis. The reaction of 2′,3′-*O*-isopropylidene-6-mercaptopurine ribonucleoside (I) with DTNB followed by phosphorylation with $POCl_3$ gave a better yield than phosphorylation of the protected mercaptopurine ribonucleoside followed by the reaction with DTNB (Scheme I).

6-(2′,3′-O-Isoproylidenepurine ribonucleoside)-5-(2-nitrobenzoic acid) Thioether (II). DTNB (10 mmoles) was suspended in 50 ml of water, and neutralized with a saturated NaOH solution. After addition of 1 mmole of isopropylidene-6-mercaptopurine ribonucleoside, the pH was adjusted to 8.0. The mixture was stirred for several days. The pH was readjusted each day, and a sample taken for paper electrophoresis at pH 6.0 in 0.01 M sodium acetate buffer. The product formed gave a new band, detected by uv absorption, approximately in the middle between DTNB in the front, and the sulfhydryl ribonucleoside. Free 2-nitro-4-mercaptobenzoic acid was in front of DTNB. Usually after 5 days, the reaction mixture was applied to a DEAE-Sephadex A-25 (HCO_3^- form) column (2.5 × 65 cm), equilibrated with CO_2-saturated water. The column was first washed with 100 ml of water, then a linear gradient, water *vs.* 0.2 M NH_4HCO_3 solution (pH 6–7, adjusted with CO_2), was applied. The total volume was 5 l. The solution in the mixing vessel was continuously saturated with CO_2. A small amount of unreacted ribonucleoside was eluted first, and after about 2 l., the thioether (II) appeared. The purity of the compound in these fractions was checked by paper electrophoresis. The fractions were lyophilized three times to remove practically all inorganic salts. The yield was 80%. For analysis the thioether was precipitated twice from pH 7.0 by acidification to pH 2.0. *Anal.* Calcd: C, 49.07; H, 3.91; N, 14.30; S, 6.54. Found: C, 47.93; H, 4.07; N, 13.89; S, 6.56. The uv spectrum of this material is shown in Figure 1. Nuclear magnetic resonance spectroscopy substantiated the presence of the purine ring (protons C_2 and C_8), of the isopropylidene ribose, and of the benzene ring of the nitrobenzoic acid.

Since formation of a thioether (II), as indicated by the elementary analysis, was unexpected, the following experiments were carried out to obtain further evidence for its structure.

Isopropylidene-6-mercaptopurine ribonucleoside (4 mmoles) was reacted with 1 mmole of [^{35}S]DTNB as described above. After 3 days excess ribonucleoside was filtered off, and the product was isolated in the usual manner. When the compound was proved pure by paper chromatography and electrophoresis, an elementary analysis was performed, and its ^{35}S was counted. The specific activity of the ^{35}S incorporated was 98–99% of the specific activity of the original DTNB. The lack of isotope dilution indicates that the 6-sulfhydryl group of the ribonucleoside was eliminated in the course of the reaction.

To prove this, 0.1 mCi of [6-^{35}S]mercaptopurine in 1 mmole was reacted with nonradioactive DTNB, by the procedure described above. The volume of solvent was doubled to accommodate the less soluble purine base. The reaction product, 6-purine-5-(2-nitrobenzoic acid) thioether, was isolated without further change. The results of elementary analysis of the chromatographically and electrophoretically pure product gave a satisfactory elementary analysis. *Anal.* Calcd: C, 45.42; H, 2.22; N, 22.07; S, 10.10. Found: C 45.24; H, 2.25; N, 21.57; S, 10.08.

The nmr spectrum again gave the expected resonance peaks of the purine protons C_2 and C_8, and of the benzene ring protons. The uv absorbancy spectrum of this component was very similar to that of the ribonucleoside thioether (see Figure 1). The mass spectrum of the compound gave a distinct peak at 317, the highest occurring mass number. The specific ^{35}S radioactivity was however only 0.03% of that of the original [6-^{35}S]sulfhydrylpurine.

When 1 mmole of the ribonucleoside thioether (II) was reacted with 3 mmoles of thioglycolic acid in 20 ml of water at pH 8.75 for 1 hr, the solution immediately turned yellow, as the free 2-nitro-4-mercaptobenzoic acid was formed. This was confirmed by paper electrophoresis at pH 6.5 in 0.01 M sodium acetate buffer, and paper chromatography in isopropyl alcohol–0.1 M Tris-acetate buffer (pH 7.5, 5:2, v/v). When ^{35}S-labeled II (see above) was reacted with thioglycolate, all of the radioactivity corresponded to the nitromercaptobenzoic acid band. The product containing the nucleoside was isolated by gradient chromatography on a DEAE-Sephadex A-25 column, as described above. It was reprecipitated several times by acidification from a solution at pH 7–8. Elementary analysis, the R_F values of the material in paper chromatography and electrophoresis, and the uv spectrum corresponded precisely to isopropylidene-6-mercaptocarboxymethylpurine ribonucleoside, prepared from the mercaptopurineisopropylidene ribonucleoside and iodoacetic acid as described in Methods.

6-(Purine 5′-ribonucleotide)-5-(2-nitrobenzoic acid) Thioether (III). Compound II (1 mmole) was dissolved in 8 ml of dried triethyl phosphate, cooled to −15° under exclusion of water, and quickly combined with 1 ml of $POCl_3$. The mixture was stirred continuously for 20 hr at 0° (see also Yoshikawa *et al.*, 1967). Barium acetate (20 ml; 1 M) was added, while the

temperature was kept below 20°. The mixture was diluted with 80 ml of water and stirred for 1.5 hr at 20°, then the free acid was neutralized to pH 8.5 with triethylamine, and the precipitate of barium phosphate was removed by centrifugation. A small amount of the isopropylidene derivative of the nucleotide (III) may be coprecipitated at this point. It may be separated from the barium phosphate by dissolving the precipitate at pH 2 and reprecipitation. The supernatants were mixed with three volumes of ethanol. After 3 hr at 0° the precipitate was washed three times each with 80% ethanol and ether and collected by centrifugation.

For removal of the isopropylidene group, the barium salt of isopropylidene III was suspended in a few milliliters of water, and mixed with an approximately equal amount of Dowex 50-X8 (200–400 mesh) in the H^+ form. The resin was packed in an 1.5 × 120 cm column. Elution with water in 10-ml fractions affords a first purification of the nucleotide, which was checked by paper electrophoresis at pH 6.0 in 0.01 M sodium acetate buffer. For final isolation the material was lyophilized and dissolved in 30 ml of an isopropyl alcohol–0.04 M NH_4HCO_3 solution (5:2, v/v, pH 6–7), and chromatographed on a cellulose column (3.5 × 150 cm), which was equilibrated with the same solvent. Small amounts of 2-nitro-4-mercaptobenzoic acid were eluted before III, which was followed by a small amount of inosinic acid. Fractions of 5 ml were collected, and the purity of the compound was checked by paper electrophoresis and paper chromatography, using the same solvent system. The solution of the purified product was concentrated with a rotatory evaporator to about one-third of the original volume, and then lyophilized. Small impurities of inorganic salts were removed by passing the material, dissolved in a small volume of water, over a Dowex 50 column. The yield was 65%. *Anal.* Calcd (monohydrate): C, 37.30; H, 3.31; N, 12.79; P, 5.65; S, 5.85. Found: C, 37.26; H, 3.26; N, 12.76; P, 5.40; S, 5.91. The product was characterized as the thioether (III) by nmr spectroscopy as described for II, and by its uv absorbancy spectrum, as the latter was practically identical with the curve shown in Figure 1.

^{32}P Labeling of the Nucleotide III. After dissolving the ribonucleoside II in triethyl phosphate and cooling the solution to −15°, following the procedure for the synthesis of unlabeled III, 0.01 ml of nonradioactive $POCl_3$ was added. The mixture was stirred at 0° for 2 hr to remove traces of water from the reaction mixture. After cooling the mixture again to −15°, 5 mCi of [^{32}P]$POCl_3$ was added and it was allowed to react for 12 hr at 0°. The reaction was brought to completion by adding 1 ml of $POCl_3$ at −15°. From this step on, the procedure described above was followed without change. The product of the reaction had a specific activity of 1.7 mCi/mmole.

Specifity of II and III as SH Reagents. Both the ribonucleoside (II) and the nucleotide thioether (III) reacted readily with aliphatic sulfhydryl compounds such as 2-mercaptoethanol, L-cysteine, or glutathione in alkaline media with release of 2-nitro-4-mercaptobenzoic acid. To prevent formation of DTNB by oxidation, the experiments were carried out under nitrogen. In the case of the reaction of the nucleoside with thioglycolic acid, the product was isolated and carefully characterized (see above). In the other cases the binding of the purine ribonucleoside or purine ribonucleotide to the aliphatic SH compound was demonstrated after separation and elution of the reaction products by paper chromatography and electrophoresis, by means of uv and nmr spectroscopy, and analysis of the phosphate content after hydrolysis of the reaction product in 2 N NaOH at 100° for 2 hr.

At pH 8, the reaction with a threefold molar excess of sulfhydryl compound was practically completed after 2 hr, whereas at pH 8.5 the reaction was completed already after 1 hr. A product with the 2-nitro-4-mercaptobenzoic acid covalently bound to the aliphatic sulfhydryl was never found. Moreover, the amount of free thiophenolate formed—as measured by the increase in absorbancy at 412 mμ (Ellman, 1959)—closely corresponded to the amount of II or III added (95–103%). The reaction of DTNB with glutathione or 2-mercaptoethanol was used as standard.

Lysine, arginine, tryptophan, serine, tyrosine, aspartic acid, and histidine were incubated at 0.1-mmole/ml concentrations, *i.e.*, a tenfold molar excess over III, in 0.1 M Tris-HCl buffer (pH 9) for 24 hr. No amino acid except cysteine was able to break the thioether bond. Neither did ammonia up to 1 M concentration and pH 12, after 6-hr incubation with II and III, form thiophenolate, or produce an additional band on paper chromatograms or electrophoreses. The reagent therefore provides a rather specific tool for affinity labeling of enzymes carrying sulfhydryl groups at or near a nucleotide binding site.

Affinity Labeling of Phosphorylase b. Crystalline phosphorylase *b* was freed from AMP as described in Methods. The enzyme (65 mg), in 2 ml of 0.01 M sodium glycerophosphate hydrochloride–0.001 M EDTA buffer (pH 8.0), was incubated with 40 mg of III for 0.5–2.0 hr at 20°. With prolonged incubation, the enzyme precipitated. At the end of the incubation period, the pH of the reaction mixture was lowered to 6.5, and it was passed over a Sephadex G-25 column (1.5 × 40 cm), equilibrated with the same buffer, to remove an excess of unreacted nucleotide. The protein was eluted and the specific enzymatic activity determined before and after addition of AMP (see Methods). An aliquot was withdrawn and the specific radioactivity was determined before and after dialysis against 0.001 M HCl for 48 hr in the cold. The enzyme solution was passed over Sephadex G-25 or over charcoal (in a 1 × 15 cm column) a second time, and the specific enzymatic activity and ^{32}P content were determined once more, the latter before and after dialysis.

Activation of phosphorylase *b* by III is shown in Figure 2, after the excess of nucleotide was removed. Additional Sephadex G-25 chromatography did not change the specific enzymatic activity or ^{32}P content of the enzyme. Furthermore, the specific radioactivity remained the same after the labeled enzyme was dialyzed for 2 days at pH 2.5 in the cold. This indicates that the activating nucleotide was covalently bound to the phosphorylase protein. In control experiments samples from the same enzyme preparation were treated in the same manner, but 5′-AMP (1×10^{-3} M) was added instead of the nucleotide reagent (III). These preparations gave the expected activity for native phosphorylase *b* in the routine assay at 30°, pH 6.8, of 70–80 μmoles of P_i/min per mg of enzymes. No radioactivity was incorporated into the protein, when a ^{35}S-labeled III (see above) was incubated with phosphorylase *b*, although the reaction resulted in 20% activation.

When 5′-AMP was added to samples of the enzyme partially activated by treatment with III at a final concentration of 1×10^{-3} M, the enzymatic activity was increased to the level of the control samples. Therefore, the enzyme was not partially denatured by reaction with III.

To obtain further evidence for a covalent attachment of the nucleotide to phosphorylase *b*, the ^{32}P-labeled enzyme was dialyzed at pH 2.5 against a large volume of cold 0.001 N HCl with two changes a day for 2 days. Peptide fingerprints of the protein were stained with ninhydrin and autoradiographed as

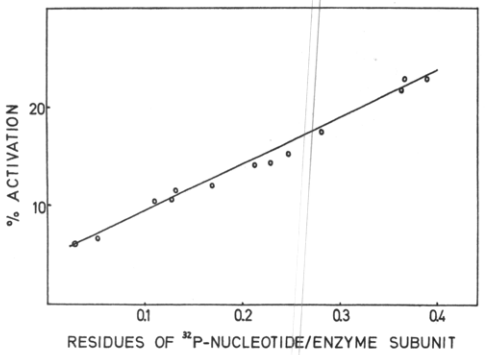


FIGURE 2: Activation of phosphorylase *b* by covalently bound nucleotide after reaction with III. Experimental details are given in the text. The ordinate denotes specific enzymatic activity as the fraction of maximal activity of the samples reached by saturation concentrations of added 5′-AMP.

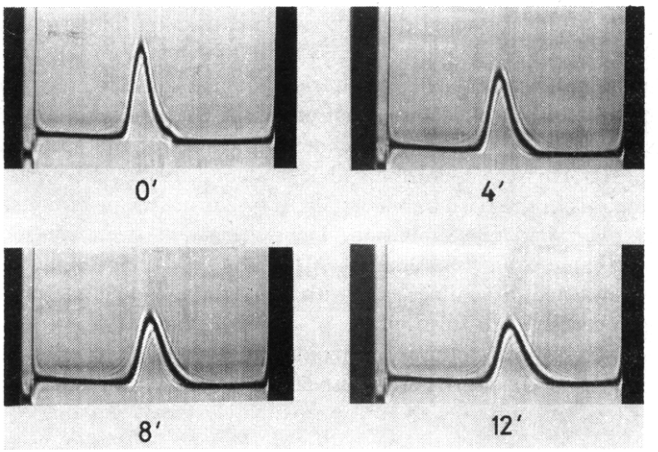


FIGURE 4: Ultracentrifugational sedimentation of nucleotide-labeled phosphorylase *b*. Experimental details see Methods. At the protein concentration used, a small amount of tetramer is observed running ahead of the dimer peak. Pictures were taken after the rotor had reached full speed (0′), and then after 4, 8, and 12 min (4′, 8′, 12′).

described in Methods. With enzyme samples activated up to 25% by reaction with III, only a single radioactive peptide was found in the autoradiograms (Figure 3). Its position was distinctly different from the position of III, II, or inosinic acid (see Figure 3). With enzymes activated to a greater extent, up to 40% by prolonged incubation with III, two weak additional radioactive spots appeared, and the main spot increased.

The $s_{20,w}$ value of the labeled phosphorylase *b* was 8.3 S, observed in sedimentation velocity experiments. The enzyme sedimented as a single symmetrical peak in the analytical ultracentrifuge (see Figure 4).

Discussion

The reaction of 6-sulfhydrylpurine and its derivatives with DTNB at pH 8.0 in aqueous solutions leads to the formation of a thioether bond linking the purine ring to one of the nitrobenzoic acid rings, and the 2-nitro-4-mercaptobenzoic acid anion is released. The long reaction time of about 5 days and the elimination of the 6-mercapto group of the purine, demonstrated by experiments with ^{35}S-labeled purine, suggested to us that the purine derivative reacts as a cationic intermediate with the disulfide bond of the DTNB, although a transition state involving tetrahedral bonding at the C-6 of the purine must also be considered. It was not possible, however, to identify the chemical state of the eliminated sulfur.

The reactivity of a mixed thioether between the position 6 of a purine derivative and a nitrobenzoate ring toward aliphatic sulfhydryl compounds can be explained on the basis of the activated state of a thioether bond involving two aromatic systems. Probably the purine base contributes to a large part to the reactivity of this bond. In contrast to a thioether bond between two aromatic rings, the new thioether formed between the purine derivative and the aliphatic amino acid residue is much more stable.

Since the thioether nucleotide does not react with amino acids other than cysteine, the reagent appears to be specific for SH groups. However, changes in reactivity of functional groups due to proximity effects in protein molecules are frequent, and reactions with other amino acid side chains can therefore not be completely excluded.

Activation of phosphorylase *b* with compound III was not carried beyond 35% (see Figure 2), because the enzyme rapidly begins to precipitate with more than one nucleotide moiety attached to it. As the peptic fingerprint and autoradiography of the labeled protein indicate, this results from side reactions of the nucleotide with the enzyme, probably with SH groups of the enzyme. At least two of these are not related to the 5′-AMP binding site (Batell *et al.*, 1968). When the activation curve in Figure 2 was extrapolated to one residue of nucleotide covalently bound per monomer, 58% activation as compared to 100% activation by addition of saturating (1×10^{-3} M) concentrations of 5′-AMP should have resulted. These calculations are based on a molecular weight corresponding to 100,000 for the monomer. In view of the rather large variety of nucleotide analogs that activate phosphorylase *b* to a greater or lesser extent aside from 5′-AMP (Okazaki *et al.*, 1968; Mott and Bieber, 1970) it is not surprising that this enzyme, with rather loose specifity for the nucleotide base, can also be partially activated by a nucleotide related to 5′-AMP which is covalently bound to its 5′-AMP site.

Most attempts to label regions of an enzyme involved in catalysis try to evaluate the modification of amino acid side chains by its inhibitory effect on the catalytic reaction. Such experiments usually entail extensive controls to exclude non-

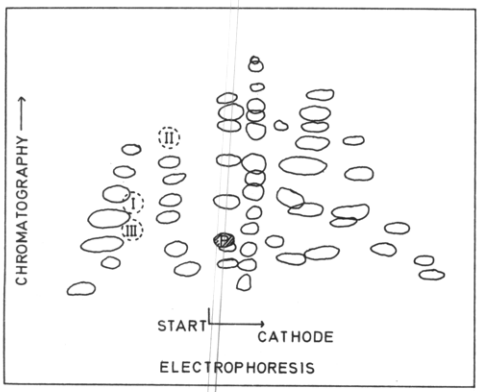


FIGURE 3: Peptic fingerprint and an autoradiogram of ^{32}P-nucleotide-labeled phosphorylase *b* (20% activation). The radioactive peptide discerned in the autoradiogram is denoted by P. Broken circles indicate the position of III, II, and inosinic acid (I) in control experiments with the same fingerprinting system, described in Methods.

specific inhibition by denaturation. Even with protein-reactive substrate analogs nonspecific inhibition must be carefully excluded by evidence for a stoichiometric relationship between incorporation of reagent and decrease of enzymatic activity.

In general, activation by a covalently bound reagent should give more confidence in a specific reaction than inhibition. This singular advantage seems to apply to the reaction of the protein reactive thioether analog of 5′-AMP and phosphorylase *b*. In view of the single labeled peptide in peptide fingerprints of the covalently activated enzyme, the possibility of a nonspecific activation seems rather remote.

In current experiments, we are trying to isolate homogeneously labeled phosphorylase *b* from the mixture of native enzyme and nucleotide derivatized enzyme after covalent activation reactions carried to about 25% of maximal enzymatic activity. This purification is an essential condition for the study of the kinetics of the labeled enzyme in comparison to 5′-AMP-activated phosphorylase *b*. Future experiments will aim at the isolation of the nucleotide-bound peptide from the peptic digest of this material for the determination of its sequence.

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A Common Intermediate in the Hydrolysis of β-Galactosides by β-Galactosidase from *Escherichia coli*†

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ABSTRACT: Kinetic data are presented which evidence the formation of a common intermediate during the hydrolysis of various aryl β-galactosides by β-galactosidase. Eight aryl β-galactosides were hydrolyzed in the presence of 0.247 M methanol. Methanol acts as a galactose acceptor in competition with water. The ratio of products (substituted phenol: methyl β-galactoside) was 3.01 ± 0.08 for these eight substrates. A similar experiment was done with four β-galactosides using 0.171 M ethanol as the acceptor. Again the ratio of products (substituted phenol:ethyl β-galactoside) was constant, 9.02 ± 0.25. If a series of substrates which vary only in the identity of the leaving group reacts in such a way as to produce a common intermediate that in turn reacts with two acceptors, water and an added acceptor, the ratio of products will be the same regardless of the leaving group. Alternatively, if no common intermediate is formed then the presence of the leaving group will influence the relative ability of two substances to serve as acceptors. Consequently, in the latter case the ratio of products will depend on the leaving group. Since a constant ratio of products was obtained with both methanol and ethanol as the extra acceptor, it may be concluded that a common intermediate is involved in the reaction mechanism for the enzymic hydrolysis of β-galactosides.

As in the case of other glycosidases, there is considerable interest in whether a common enzymic intermediate is formed during the hydrolysis of different galactosides by β-galactosidase. In nonenzymic hydrolysis of glycosides the nature of the intermediate depends on the conditions of the hydrolysis. In acid the hydrolysis proceeds through a carbonium ion intermediate (BeMiller, 1967), whereas in base an intramolecular displacement reaction probably occurs (Ballou, 1954; Gasman and Johnson, 1966; Piszkiewicz and Bruice, 1968). Both these processes seem to have analogous counterparts in enzymic systems. Thus a stabilized carbonium ion is favored

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