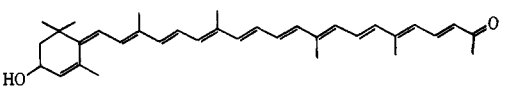
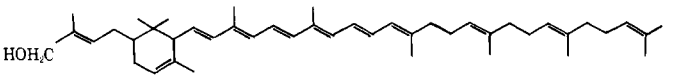
3-Hydroxy-5′-methyl-4,5′-*retro*-5′-apo-β-caroten-5′-one or 3-hydroxy-4,5′-*retro*-5′-apo-β-caroten-5′-one



Tangeraxanthin

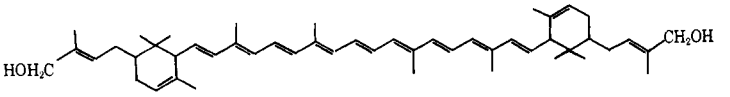
Higher Carotenoids

2-(4-Hydroxy-3-methyl-2-butenyl)-7′,8′,11′,12′-tetrahydro-ε,ψ-carotene



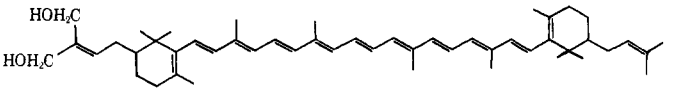
Nonaprenoxanthin

2,2′-Bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene



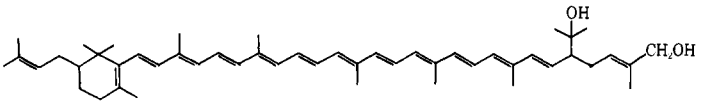
Decaprenoxanthin

2-[4-Hydroxy-3-(hydroxymethyl)-2-butenyl]-2′-(3-methyl-2-butenyl)-β,β-carotene



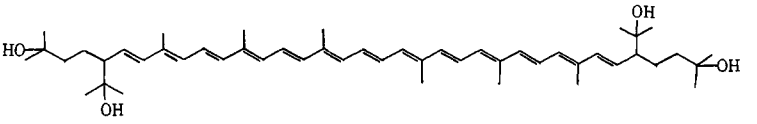
C.p. 450

2′-(4-Hydroxy-3-methyl-2-butenyl)-2-(3-methyl-2-butenyl)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-1′-ol



C.p. 473

2,2′-Bis(3-hydroxy-3-methylbutyl)-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol



Bacterioruberin

Pyridoxal 5′-Phosphate as a Site-Specific Protein Reagent for a Catalytically Critical Lysine Residue in Rabbit Muscle Phosphoglucose Isomerase*

Klaus D. Schnackerz† and Ernst A. Noltmann‡

ABSTRACT: Rabbit muscle phosphoglucose isomerase has been found to be inhibited by pyridoxal 5′-phosphate at concentrations as low as 0.2 mM. When tested in the presence of substrate, this inhibition is competitive, with a K_i of 2.4 mM. The inhibition can be changed to an irreversible inactivation by reduction of the enzyme–pyridoxal 5′-phosphate complex with sodium borohydride. Both a substrate equilibrium mixture and 6-phosphogluconate (a competitive inhibitor of phosphoglucose isomerase) protect the enzyme against this inactivation. The reaction of pyridoxal 5′-phosphate with phosphoglucose isomerase is highly specific since free pyridoxal, 5-deoxypyridoxal, and other aldehydes showed an inhibitory effect only at concentrations up to several 100-fold higher than required for pyridoxal 5′-phosphate. The unreduced phosphoglucose isomerase–pyridoxal 5′-phosphate complex displays a difference spectrum with a maximum near 430 nm, typical for Schiff base formation that accompanies pyridoxal 5′-phosphate–protein interaction. Also, after reduction by sodium borohydride, the absorption spectrum shows a maximum at 325 nm characteristic for ε-aminophosphopyridoxyllysine. Finally, N^6-pyridoxyllysine has been identified in acid hydrolysates of the reduced phosphoglucose isomerase–pyridoxal 5′-phosphate complex. The high degree of specificity permitted the use of pyridoxal 5′-phosphate, in combination with reduction by sodium borohydride, as a stoichiometric inhibition titrant. This approach yielded a stoichiometry of 2 mole equiv of pyridoxal 5′-phosphate bound per mole of enzyme, extrapolating to 100% loss of enzymatic activity. The results are interpreted as lending strong support to the previously proposed involvement of a critical ε-aminolysyl group in the phosphoglucose isomerase reaction.

Studies dealing with the effect of pH and temperature on the kinetic parameters of rabbit muscle phosphoglucose isomerase have recently led to a proposal for the reaction mechanism of this enzyme in which both a histidine and a lysine residue participate in the catalytic process (Dyson and Noltmann, 1968). Ultraviolet difference spectroscopy, performed

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as a function of pH, subsequently indicated that no major conformational changes occurred at either pH 6.75 or 9.3 (Dyson and Noltmann, 1969a,b), thus supporting the original contention that the residues identified by the corresponding pK values are in fact involved in the catalytic functioning of the enzyme. Chemical modification of the enzyme protein, resulting in loss of enzymatic activity, by dye-sensitized photooxidation (Chatterjee and Noltmann, 1967a), reaction with organic mercurials (Chatterjee and Noltmann, 1967b), or carboxamidomethylation (Schnackerz and Noltmann, 1970) further affirmed the role of histidine and, at the same time, rendered cysteine and methionine unlikely as catalytically critical amino acid residues. Especially the alkylation experiments (Schnackerz and Noltmann, 1970), in which full protection of histidine from carboxamidomethylation and complete preservation of enzymatic activity was found on addition of 6-phosphogluconate (a competitive inhibitor of phosphoglucose isomerase), were in support of the argument for participation of this residue. The same technique, however, proved less satisfactory for the chemical identification of a lysine residue, making it necessary to seek another approach.

Fischer and collaborators (1958) had shown that pyridoxal 5′-phosphate is an integral part of rabbit skeletal muscle phosphorylase *b* and that it is bound to the enzyme as a substituted aldimine derivative which is converted to a free Schiff base by treatment with acid, base, or urea. These workers had also demonstrated by reduction of the Schiff base with sodium borohydride that the binding of pyridoxal 5′-phosphate to the enzyme occurs *via* the ε-amino group of a lysine residue.

More recently, Rippa *et al.* (1967) made the interesting discovery that pyridoxal 5′-phosphate, in combination with reduction by sodium borohydride, can also be used to identify reactive lysine residues of other enzymes which *per se* have no requirement for this factor. This approach led to the demonstration of a critical lysine residue in *Candida utilis* 6-phosphogluconate dehydrogenase (Rippa *et al.*, 1967; Rippa and Pontremoli, 1969) and in rabbit muscle aldolase (Shapiro *et al.*, 1968). The present investigation was therefore initiated to test the suitability of pyridoxal 5′-phosphate as a site-specific protein reagent for the catalytically critical lysine residue postulated for rabbit muscle phosphoglucose isomerase.[1] Numerous other reports have now appeared in the literature which confirm the usefulness of pyridoxal 5′-phosphate as a tool for the identification of lysine residues (*e.g.*, Kaldor and Weinbach, 1966; Anderson *et al.*, 1966; Marcus and Hubert, 1968; Ronchi *et al.*, 1969; Benesch *et al.*, 1969; Domagk *et al.*, 1969; Uyeda, 1969; Johnson and Deal, 1970; Piszkiewicz *et al.*, 1970; Forcina *et al.*, 1971; Shematek and Arfin, 1971). Moreover, while testing the effect of pyridoxal 5′-phosphate on various glycolytic enzymes, Domschke and Domagk (1969) have also observed inhibition of yeast phosphoglucose isomerase.

Materials and Methods

Enzymes. Phosphoglucose isomerase was isolated from rabbit muscle according to the method previously described (Noltmann, 1964, 1966) and crystallized five times from neutralized ammonium sulfate solutions. Some of the initial experiments were conducted with a preparation having a specific activity of 600 units/mg. All other preparations had specific activities of 750–850 μmoles of fructose 6-phosphate isomerized to glucose 6-phosphate per min at 30°. Prior to each series of experiments, an aliquot of the crystalline suspension was centrifuged at 0° for 60 min at 35,000*g* and the pellet dissolved in the same medium used for subsequent dialysis. Protein concentrations were determined spectrophotometrically with use of an absorptivity coefficient of 1.32 (10-mm light path) at 280 nm for a solution containing 1 mg of enzyme/ml (Noltmann, 1966). Molar concentrations of the enzyme were calculated on the basis of a molecular weight of 132,000 (Pon *et al.*, 1970). Glucose-6-phosphate dehydrogenase from yeast and 6-phosphogluconate dehydrogenase were purchased from Boehringer Mannheim Corp.

Substrates and Reagents. The sodium salts of glucose 6-phosphate, fructose 6-phosphate, 6-phosphogluconate, and nicotinamide–adenine dinucleotide phosphate were obtained from Boehringer Mannheim Corp. Solutions of these compounds were standardized by enzymatic assay with an absorptivity coefficient of 6.22×10^3 M^{-1} cm^{-1} at 340 nm for NADPH. Sodium borohydride was obtained from Metal Hydrides Inc., Beverly, Mass., and Serva, Heidelberg, Germany. The actual concentrations of sodium borohydride in solution and its stability were determined by a volumetric method according to which sodium borohydride is oxidized with excess potassium iodate and the unreduced iodate is determined by iodometric titration (Lyttle *et al.*, 1952). Sephadex G-25, G-50, and G-75 were purchased from Pharmacia; octyl alcohol from Matheson; poly-L-lysine hydrobromide (mol wt *ca.* 150,000) from Sigma. Pyridoxal hydrochloride and pyridoxal 5′-phosphate monohydrate were obtained from Calbiochem or from Serva, Heidelberg. Pyridoxal 5′-phosphate, tritium labeled in the 4′ position, prepared according to the method of Stock *et al.* (1966), was a gift from Mr. Ehrlich of Dr. E. Helmreich's laboratory. Concentrations of pyridoxal and pyridoxal 5′-phosphate were determined spectrophotometrically in 0.1 M NaOH with molar extinction coefficients of 5800 M^{-1} cm^{-1} at 300 nm for the former and 6600 M^{-1} cm^{-1} at 388 nm for the latter (Peterson and Sober, 1954). 5-Deoxypyridoxal was prepared by the method of Iwata (1968) and measured spectrophotometrically according to Nakamoto and Martell (1959). Pyridine (Mallinckrodt) was distilled over ninhydrin prior to use as an electrophoresis solvent. All other chemicals were of the best grade commercially available and were used without further purification.

Preparation of N^{ϵ}-Pyridoxyllysine. Poly-L-lysine hydrobromide (20 mg) was treated with pyridoxal (0.38 mmole) in 50 mM sodium acetate (pH 6.0). The reaction mixture was allowed to stand for 10 min at 0° and was then reduced with sodium borohydride as described below. Low molecular weight reactants were removed by dialysis against 50 mM sodium acetate (pH 6.0). The product showed a major absorption band at 325 nm, characteristic for the reduced Schiff base (Fischer *et al.*, 1963). Hydrolysis of a small sample in 6 N HCl for 20 hr at 110° revealed that not all of the ε-amino groups had reacted. After dialysis against distilled water and lyophilization, the remaining polymer was again treated with 0.38 mmole of pyridoxal, reduced with borohydride, and hydrolyzed. The hydrolysate was taken to dryness under reduced pressure, dissolved in distilled water, and again taken to dryness. This process was repeated several times.

Enzyme Assay. Enzyme activity was measured in a coupled spectrophotometric assay in which nicotinamide dinucleotide phosphate and glucose-6-phosphate dehydrogenase were employed as auxiliary components (Noltmann, 1964). Units are expressed as micromoles of substrate turned over per minute

[1] A preliminary report on part of this work has been given earlier (K. D. Schnackerz and E. A. Noltmann, Abstracts of the Pacific Slope Biochemical Conference, Seattle, Wash., June 1969, p 19).

at 30°. In cases where high concentrations of pyridoxal 5′-phosphate were used, glucose-6-phosphate dehydrogenase concentrations had to be increased accordingly to compensate for the inhibition of the latter by pyridoxal 5′-phosphate. Such inhibition has been observed independently by Domagk *et al.* (1969).

Standard Procedure for Reduction with Sodium Borohydride. Enzyme (2–4 mg/ml) was incubated for 10 min at 0° in 27 mM sodium acetate (pH 6.0) with various amounts of pyridoxal 5′-phosphate ranging from 5 to 580 μM. A 5-μl aliquot was withdrawn to determine the initial enzyme activity. Octyl alcohol was added in order to avoid foaming. A freshly prepared solution of sodium borohydride (0.6 M), dissolved in distilled water or 0.001 N sodium hydroxide to prevent acid-catalyzed decomposition, was added in 10-μl portions totalling a 50- to 75-fold excess with respect to the highest pyridoxal 5′-phosphate concentration used. After each addition of sodium borohydride the pH was readjusted to 6.0 with 1 N acetic acid. The enzymatic activity was monitored at least twice during the reduction phase and also after completion of the experiment. Following reduction of the enzyme–pyridoxal 5′-phosphate complex the reaction mixture was passed through a Sephadex column (G-25, fine; 20 mm diameter) jacketed to keep the temperature at 5°. For each column 4 g of the Sephadex, equilibrated with 0.05 M sodium acetate (pH 6.0), was used to produce a column of 50-mm height. A flow rate of 2 ml/min was maintained with a peristaltic pump and the column effluent was monitored at 280 nm with a LKB Uvicord II photometer. Maximally 20 min were required from the first addition of borohydride to complete separation of the reaction products. As alternative methods for removal of excess reagents dialysis against three changes of a 3000-fold volume each of 0.05 M sodium acetate (pH 6.0) or precipitation with ammonium sulfate were used occasionally. It should be noted that dialysis prior to the precipitation is advantageous since the presence of octyl alcohol may make it difficult to collect the precipitate by centrifugation. During the entire reduction procedure the sample was exposed to light as little as possible.

The amount of pyridoxal 5′-phosphate bound per mole of enzyme was determined spectrophotometrically by assuming a value of 10,000 for the molar absorptivity coefficient of N^6-phosphopyridoxyllysine at 325 nm (Forrey *et al.*, 1971). Special precautions were taken to obtain measurements as precise and reproducible as possible by using the 0.1-*A* scale of a Cary Model 15 spectrophotometer. Best reproducibility was achieved through use of a tungsten lamp with the voltage adjusted to 90 V.

Measurement of Radioactivity. For liquid scintillation counting 500-μl samples of the Sephadex column effluent mixed with Bray scintillation fluid (Bray, 1960) were counted in a Packard Model 3380 TriCarb spectrometer. The specific radioactivity of the tritiated pyridoxal 5′-phosphate used was 9475 dpm/nmole.

Identification of N^6-Pyridoxyllysine after Reaction of Phosphoglucose Isomerase with Pyridoxal 5′-Phosphate. The reduced pyridoxal 5′-phosphate–enzyme complex was dialyzed for 12 hr in the dark against several changes of distilled water and then hydrolyzed in sealed tubes in the presence of 6 N HCl for 20 hr at 110°. The hydrolysate was concentrated under reduced pressure, dissolved in water, and lyophilized. This procedure was repeated several times to free the product completely from residual HCl. Aliquots of the hydrolysate were applied to sheets of Whatman No. 3MM paper and subjected to high-voltage electrophoresis in three systems: (1) pyridine–acetic acid–water (1:10:89, v/v), pH 3.5, for 60 min at 2000 V; (2) 1.5 M formic acid, pH 1.6, for 30 min at 2500 V; (3) 0.04 M potassium phosphate, pH 7.5, for 45 min at 3000 V. After drying of the paper sheets a fluorescent spot was visible under uv light with a mobility in each solvent system identical with that of the synthetic pyridoxyllysine.

TABLE I: Inactivation of Rabbit Muscle Phosphoglucose Isomerase by Pyridoxal 5′-Phosphate and Other Aldehydes after Treatment with Sodium Borohydride.[a]

Inhibitor	Concn of Inhibitor (mM)	% Enzyme Act. Remaining
None		100
Pyridoxal 5′-phosphate	0.19	18
Pyridoxal	8.4	84
5-Deoxypyridoxal	5.5	30
Acetaldehyde	75	25
Acetone	75	69
Propionaldehyde	107	46
Isobutyraldehyde	85	82
Isovalderaldehyde	72	32
Cyclohexanone	74	94

[a] Conditions: 0.027 M sodium acetate (pH 6.0); temperature, 0°; enzyme, 2.3 mg/ml, except for 5-deoxypyridoxal, 4.0 mg/ml. The enzyme was incubated for 10 min with the inhibitor prior to the addition of sodium borohydride.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed at 10° in a Spinco Model E analytical ultracentrifuge equipped with a RTIC unit and a photoelectric scanning device. Double-sector cells with 12-mm filled Epon centerpieces were employed in an An-D rotor at 40,000 rpm. Enzyme samples were dialyzed against several changes of 0.1 M sodium phosphate (pH 7.1) prior to ultracentrifugal analysis.

Results

Effect of Pyridoxal 5′-Phosphate and Some Unphosphorylated Aldehydes on the Enzymatic Activity. When rabbit muscle phosphoglucose isomerase was incubated with low concentrations of pyridoxal 5′-phosphate and then treated with an excess of sodium borohydride, enzyme activity was lost—the extent depending upon the concentration of pyridoxal 5′-phosphate added—whereas treatment with sodium borohydride alone did not show any effect. The specificity of this inactivation by the phosphorylated pyridoxal is most apparent when compared to the effect produced by other aldehydes. As shown in Table I, pyridoxal itself, even at a 40-fold higher concentration than pyridoxal 5′-phosphate, yielded only 16% inhibition compared with the 82% achieved by 0.2 mM pyridoxal phosphate. 5-Deoxypyridoxal at 5.5 mM caused 70% inhibition of phosphoglucose isomerase. Other aldehydes tested required several 100-fold concentrations over that of the phosphorylated pyridoxal to produce an inhibitory effect (Table I). When tested in the absence of treatment with sodium borohydride, pyridoxal 5′-phosphate was found to be a competitive inhibitor of phosphoglucose isomerase with a K_i value of 2.4 mM.

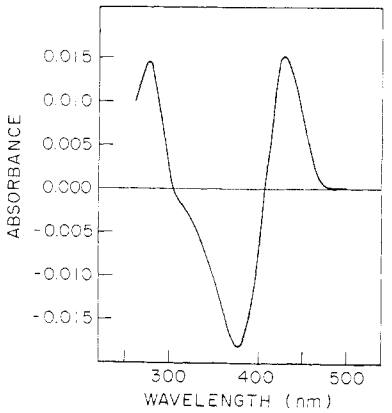

FIGURE 1: Difference spectrum of the phosphoglucose isomerase–pyridoxal 5′-phosphate complex. Spectral measurements were made with cylindrical tandem cells (Hellma No. 235/10) in a Cary Model 15 spectrophotometer. The sample cell contained 0.44 mg of rabbit muscle phosphoglucose isomerase per ml plus 0.1 mM pyridoxal 5′-phosphate in 0.05 M sodium acetate (pH 6.0) in one compartment and buffer only in the other, whereas in the reference cell, enzyme and pyridoxal 5′-phosphate were in separate compartments. The difference spectra were determined before and after addition of pyridoxal 5′-phosphate to allow for appropriate base-line correction. The spectrum shown was taken 60 min after the addition of pyridoxal 5′-phosphate.

Since it was not possible to inactivate the enzyme completely in a single step, no matter how high the pyridoxal 5′-phosphate concentration was, treatment with pyridoxal phosphate and sodium borohydride was repeated several times. After each cycle the reduced pyridoxal 5′-phosphate–enzyme complex was separated from excess reagents by gel filtration on Sephadex G-75, followed by dialysis against the same sodium acetate buffer. Three such inactivation cycles were usually sufficient to decrease enzyme activity to below 5% of its initial value (Table II). Preincubation times longer than 10 min did not significantly increase the extent of inactivation. Also, no difference was observed in the degree of inactivation when the enzyme was preincubated with pyridoxal phosphate at 30° or at 0°. Therefore, 10-min preincubation at 0° in sodium acetate buffer (pH 6.0) was chosen as standard condition.

Protection by Substrate and Substrate Analogs. If reaction with pyridoxal 5′-phosphate were to occur at a lysine residue that is essential for the catalytic functioning of the enzyme, substrate or a substrate analog would be expected to have an effect on the inactivation produced by the pyridoxal phosphate–sodium borohydride treatment. Such data are presented in Table III indicating that substrate as well as the competitive inhibitor 6-phosphogluconate can almost completely protect from reaction with pyridoxal phosphate.

TABLE II: Inactivation of Rabbit Muscle Phosphoglucose Isomerase by Repeated Treatments with Pyridoxal 5′-Phosphate–Sodium Borohydride.[a]

Inactivation Cycle	Sp Act. (Units/mg)
Before first	574
First	116
Second	76
Third	23

[a] Conditions: 0.027 M sodium acetate, pH 6.0; temperature, 0°; initial enzyme concentration, 2.3 mg/ml. The enzyme was incubated for 10 min with a 200-fold molar excess of pyridoxal 5′-phosphate (0.47 mM) prior to the addition of sodium borohydride. The reaction mixture was subjected to gel filtration and dialysis after each cycle.

TABLE III: Protection of Rabbit Muscle Phosphoglucose Isomerase against Inactivation by Pyridoxal 5′-Phosphate–Sodium Borohydride Treatment.[a]

Protectant	Concn of Protectant (mM)	% Enzyme Act. Remaining
Control (without $NaBH_4$)		100
None		20
Fructose 6-phosphate[b]	0.51	81
Fructose 6-phosphate[b]	1.02	93
6-Phosphogluconate	0.53	95

[a] Conditions: 0.027 M sodium acetate, pH 6.0; temperature, 0°; enzyme, 2.3 mg/ml; pyridoxal 5′-phosphate, 0.45 mM; 10-min incubation of pyridoxal 5′-phosphate and protectant prior to the addition of sodium borohydride. [b] Immediately on addition, fructose 6-phosphate will be converted to an equilibrium mixture of glucose 6-phosphate and fructose 6-phosphate.

Difference Spectrum of the Pyridoxal 5′-Phosphate–Phosphoglucose Isomerase Complex. Addition of pyridoxal 5′-phosphate to phosphoglucose isomerase in sodium acetate (pH 6.0) resulted in characteristic spectral changes typical for this kind of protein–ligand interaction. Positive absorption bands were found at 432 and 278 nm and a negative absorption band at 378 nm with a shoulder in the 340-nm region (Figure 1). With time the difference spectrum underwent changes only in amplitude; the peak at 432 nm increased slightly whereas the trough at 378 nm was considerably enlarged when spectra obtained 5 min and 1 hr after addition of pyridoxal 5′-phosphate were compared. Absorption maxima in the 430-nm region have been found in all pyridoxal 5′-phosphate–protein complexes and are attributed to a form in which the phenolic hydroxyl group is hydrogen bonded to the amino nitrogen of the Schiff base (Metzler, 1957; Jenkins and Sizer, 1957). The shoulder in the 340-nm region, which is much more pronounced at high pH values, is believed to represent an aldimine without intramolecular hydrogen bonding (Morino and Snell, 1967).

Spectral Properties of the Reduced Pyridoxal 5′-Phosphate–Enzyme Complex. When the pyridoxal 5′-phosphate–enzyme complex was reduced with sodium borohydride, the spectrum showed the typical absorption of the protein at 280 nm and, in addition, that of bound pyridoxal phosphate with a maximum at 325 nm (Figure 2). This absorption maximum at 325 nm is characteristic of the formation of ϵ-aminophosphopyridoxyllysine, the molar absorptivity coefficient of which was determined to be 10,000 by Forrey *et al.* (1971). In the experiment shown in Figure 2 an excess of pyridoxal phosphate had been used, which resulted in the binding of 2.5 moles/mole of enzyme.

Correlation between Enzymatic Activity and Moles of Pyri-

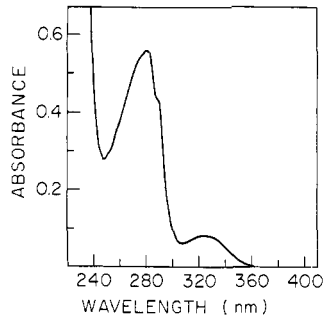

FIGURE 2: Absorption spectrum of rabbit muscle phosphoglucose isomerase after treatment with pyridoxal 5′-phosphate and sodium borohydride. Enzyme, 2.3 mg/ml; pyridoxal 5′-phosphate, 2.45 mM. Specific activity was 860 and 82 μmoles per min per mg, respectively, before and after treatment with sodium borohydride. The ratio of bound pyridoxal 5′-phosphate to enzyme was 2.5. The spectrum was taken after gel filtration and exhaustive dialysis against 50 mM sodium acetate (pH 6.0).

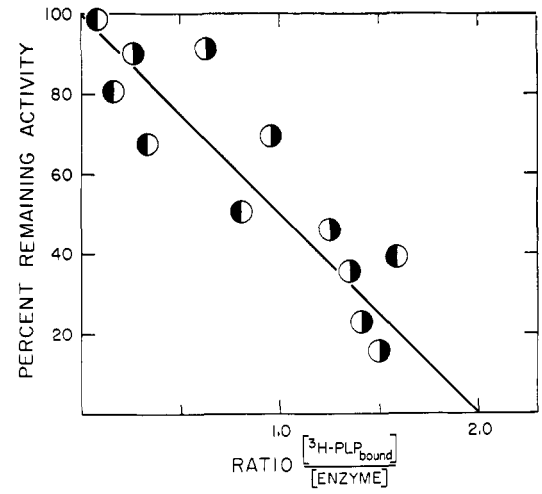

FIGURE 3: Stoichiometry of the reaction between [^{3}H]pyridoxal 5′-phosphate and rabbit muscle phosphoglucose isomerase. Enzyme concentration, 2.3 (◐) or 4.0 mg per ml (◑); [^{3}H]pyridoxal 5′-phosphate (4.27 mCi/mmole), concentrations ranging from 5 to 580 μM; sodium acetate, pH 6.0, 0.027 M; temperature 0°. The enzyme was incubated for 10 min with pyridoxal 5′-phosphate prior to the addition of sodium borohydride. After complete reduction, residual enzyme activity was determined. Following gel filtration of each sample, the radioactivity incorporated into the protein moiety was measured and the ratio of pyridoxal 5′-phosphate bound to enzyme calculated. The straight line drawn represents a least-squares fit of all points.

doxal 5′-Phosphate Bound per Mole of Phosphoglucose Isomerase.[2] Preliminary experiments made it likely that pyridoxal 5′-phosphate might be utilized as a "titrant" for "stoichiometric inhibition titration" (Mahowald *et al.*, 1962) of phosphoglucose isomerase. The enzyme was preincubated with increasing concentrations of tritium-labeled pyridoxal phosphate (4.27 mCi/mmole) and the enzymic activity determined. After exposure to pyridoxal phosphate for 10 min and reduction with sodium borohydride, excess reagents were removed by passing the samples through Sephadex (G-25, fine). The amount of pyridoxal 5′-phosphate bound was then determined spectrophotometrically at 325 nm as well as by measuring the radioactivity incorporated into the protein moiety. When the residual enzyme activity is plotted as a function of moles of pyridoxal phosphate bound per mole of enzyme either in terms of tritium label incorporated (Figure 3) or based on the absorption at 325 nm (Figure 4), 100% loss of activity extrapolates ("titrates") to 2.0 and 2.4 equiv, respectively, of pyridoxal phosphate bound per mole of enzyme. The data shown in both figures are the results of two independent experiments obtained with different enzyme preparations. In all experiments, in which the enzyme was exposed only once to pyridoxal 5′-phosphate, a residual activity of 10–15% of that of the native enzyme always remained.

Isolation and Identification of N^6-Pyridoxyllysine from the Reduced Pyridoxal 5′-Phosphate–Phosphoglucose Isomerase Complex. The inactive reduced enzyme–pyridoxal 5′-phosphate complex was dialyzed exhaustively against distilled water and then hydrolyzed with HCl, as described under Methods. N^6-Pyridoxyllysine was then identified in the acid hydrolysate by means of paper electrophoresis. In three different electrophoretic systems, a single fluorescent spot was observed with a mobility identical with that of synthetic N^6-pyridoxyllysine. The ultraviolet-sensitive compound could be stained with ninhydrin, confirming the presence of a free amino group. The fluorescence of both the synthetic product and the protein derivative was found to be equally quenched by ammonia vapor.

Sedimentation Analysis of the Pyridoxal 5′-Phosphate–Phosphoglucose Isomerase Complex. To investigate whether gross structural changes had occurred as a result of the modification, an enzyme sample containing 1.4 mole equiv of pyridoxal 5′-phosphate was subjected to ultracentrifugation under the conditions described in the Methods section. At a protein concentration of 0.6 mg/ml, a sedimentation coefficient of 4.6 S was found which is in good agreement with the value of 4.98 S reported previously for $s^0_{10°,P}$ of native rabbit muscle phosphoglucose isomerase (Pon *et al.*, 1970). It can therefore be concluded that modification by pyridoxal 5′-phosphate does not cause dissociation of the enzyme into subunits.

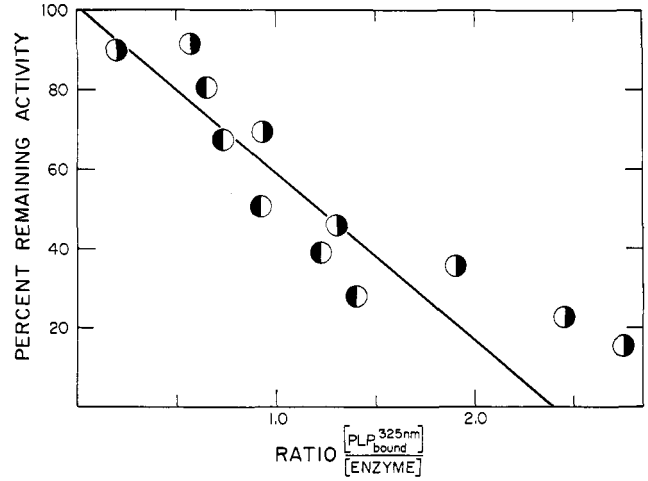

FIGURE 4: Stoichiometry of the reaction between pyridoxal 5′-phosphate (A_{325} nm) and rabbit muscle phosphoglucose isomerase. Conditions were the same as described in the legend to Figure 3 except that after gel filtration the amount of pyridoxal 5′-phosphate bound was determined from the absorbance at 325 nm with use of a value of 10,000 for the molar absorptivity coefficient (see text).

[2] These experiments were performed in collaboration with P. Bruch and were supported by a research grant from the Deutsche Forschungsgemeinschaft to K. D. S.

Discussion

A comparison of the data concerning the inhibition of rabbit muscle phosphoglucose isomerase by pyridoxal 5′-phosphate and by other, nonphosphorylated aldehydes clearly warrants the conclusion that the reaction with pyridoxal 5′-phosphate is very specific. Pyridoxal and deoxypyridoxal which are structurally related to pyridoxal 5′-phosphate show a much smaller degree of inactivation after treatment with sodium borohydride and substantially higher concentrations are required compared with pyridoxal phosphate. It should be noted that in acidic and neutral solutions pyridoxal is present largely in its hemiacetal form whereas pyridoxal phosphate is 100% aldehyde (Metzler and Snell, 1955; Nakamoto and Martell, 1959). The inactivation produced by deoxypyridoxal is therefore a more direct measure for assessing the effect of the phosphate group in pyridoxal phosphate.

From the almost complete protection afforded by relatively low concentrations of either substrate or 6-phosphogluconate against inactivation produced by the pyridoxal phosphate–sodium borohydride treatment it may also be inferred that the modification does, in fact, occur at the same site responsible for binding of the substrate or the competitive inhibitor. Equally, the linear proportionality between loss of enzyme activity and formation of the pyridoxal phosphate–phosphoglucose isomerase complex is most compatible with the involvement of a catalytically critical lysine residue. Stoichiometric inhibition titration (Mahowald *et al.*, 1962) of the enzymatic activity is not likely to occur if the residue undergoing reaction is merely concerned with stabilizing the active conformation of the protein. The evidence presented here would thus appear to be a clear indication that pyridoxal 5′-phosphate acts on phosphoglucose isomerase as a site-specific reagent and that not only its aldehyde group is involved in the formation of the Schiff base, but also that the phosphate group in the 5′ position is essential for binding, perhaps in a manner analogous to that of the natural substrate.

The results of the stoichiometry experiments analyzed by two independent methods indicate that complete loss of enzymatic activity extrapolates to 2 moles of pyridoxal 5′-phosphate bound per mole of phosphoglucose isomerase. The fact that the observed stoichiometry is so close to a small, integral number is again good evidence for the specificity of the reaction in that particular, critical lysine residues are involved. Since rabbit muscle phosphoglucose isomerase is composed of two identical or very similar subunits (Blackburn *et al.*, 1970; Blackburn, 1971; *cf.* also Yoshida and Carter, 1969), the stoichiometry of 2 in terms of the native enzyme (mol wt, 132,000) therefore implies that the enzyme has one active site per subunit. The similarity of the results regarding the stoichiometry as measured by two independent methods, *i.e.*, uv spectroscopy and radioactive labeling, allows furthermore the conclusion that the extinction coefficient of enzyme-bound pyridoxal phosphate is equal or at least quite similar to that of free phosphopyridoxyllysine and that environmental factors such as reported for phosphorylase *b* (Cortijo and Shaltiel, 1970) do not appear to have a significant effect on pyridoxal 5′-phosphate bound to rabbit muscle phosphoglucose isomerase.

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Catalytic Versatility of Erythrocyte Carbonic Anhydrase. IX. Kinetic Studies of the Enzyme-Catalyzed Hydrolysis of 3-Pyridyl and Nitro-3-pyridyl Acetates*

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ABSTRACT: The present investigation demonstrates that bovine carbonic anhydrase powerfully catalyzes the hydrolysis of 3-acetoxypyridine, 3-acetoxy-2-nitropyridine, and 3-acetoxy-2,6-dinitropyridine. The reactions follow Michaelis–Menten kinetics over the whole range of substrate concentrations studied, including the region [S] > K_m. The pH–activity profiles are approximately sigmoid; the esterase activity is very small below pH 6 and rises to an intermediate plateau above pH 8. The inflection point at 25.0° lies at pH 7.56 for 3-acetoxy-2-nitropyridine and pH 7.4 for 3-acetoxy-2,6-dinitropyridine. A more detailed treatment of the data shows that for all these substrates the mild variation of K_m with pH is dictated by the respective turnover number (k_2) while the formal binding constants (k_1/k_{-1}) are nearly independent of pH in the range 6.0–8.0. A comparison of these kinetic parameters suggests that when the first nitro group is incorporated into the ester it leads to an increase in esterase activity through both a larger turnover number and a better binding; of these, the former factor predominates. However, the incorporation of the second nitro group into the ester leads only to an increase in the turnover number but leaves the formal binding constant, k_1/k_{-1}, essentially unchanged. The relatively high solubility of these esters makes them ideal substrates for the kinetic identification of transient acyl-enzyme intermediates preceding a slower turnover reaction. However, throughout this study, no evidence could be obtained of an initial "burst" of release of either 3-hydroxy-2-nitropyridine or of 3-hydroxy-2,6-dinitropyridine even under highly favorable conditions. Acetazolamide is a powerful inhibitor of esterase activity. The K_i values at pH 7.47 and 25.0° are: 2.8×10^{-7} M with 3-acetoxypyridine, 2.9×10^{-8} M with 3-acetoxy-2-nitropyridine, and 2.0×10^{-8} M with 3-acetoxy-2,6-dinitropyridine. The inhibitions appear to be noncompetitive.

It has been demonstrated in these laboratories that erythrocyte carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) (CA)[1] is not, as has been previously thought, an absolutely specific catalyst for the reversible hydration of carbon dioxide, a catalysis that does indeed embody its physiological function, but the enzyme also powerfully catalyzes the reversible hydration of various aldehydes (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Dickerson, 1968), and the hydrolysis of nitrophenyl esters (Pocker and Stone, 1965, 1967; Pocker and Storm, 1968; *cf.* also Tashian *et al.*, 1964; Malmström *et al.*, 1964; Armstrong *et al.*, 1966). This versatility was later shown to include also the hydrolysis of sulfonate esters (Lo and Kaiser, 1966; Y. Pocker and S. Sarkanen, unpublished observations) and of 1-fluoro-2,4-dinitrobenzene (Henkart *et al.*, 1968). More recently it has been shown that the enzyme also catalyzes the dehydration of the 2,2-dihydroxypropionate anion to pyruvate ion (Pocker and Meany, 1970). These findings, coupled with the fact that the experimental techniques necessary to obtain accurate rate data for many of these substrates are far simpler than those used in the study of the reversible hydration of carbon dioxide, have allowed a more comprehensive study of this enzyme and a fuller appraisal of its mode of action.

Kinetics studies on propionaldehyde, isobutyraldehyde, and pivaldehyde hydrations revealed that the catalytic efficiency of bovine carbonic anhydrase (BCA) with respect to these substrates was inversely proportional to the size of the aldehyde (Pocker *et al.*, 1965). Furthermore, a comparison of the various homologous series of *p*-nitrophenyl esters re-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; *p*-NPA, *p*-nitrophenyl acetate; AP, 3-acetoxypyridine; ANP, 3-acetoxy-2-nitropyridine; ADNP, 3-acetoxy-2,6-dinitropyridine.