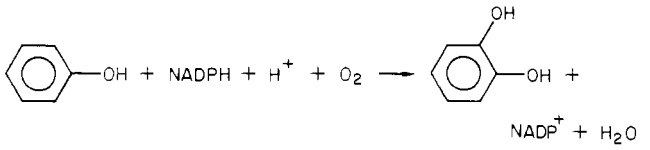
Phenol Hydroxylase from Yeast: A Lysyl Residue Essential for Binding of Reduced Nicotinamide Adenine Dinucleotide Phosphate†

Halina Y. Neujahr* and Karin G. Kjellén

ABSTRACT: The inducible enzyme phenol hydroxylase from *Trichosporon cutaneum* is a FAD-containing monooxygenase which catalyzes the NADPH-dependent hydroxylation of phenol to catechol. The enzyme contains 16 cysteinyl residues, 6–8 of which are essential for retention of FAD and for activity. The complete amino acid composition is now reported as well as the results of studies with amino group reagents. A number of amino group reagents inhibit the enzyme severely, most of them with a concomitant, more or less extensive release of FAD. P-pyridoxal inhibits the enzyme specifically, without affecting its FAD content. The P-pyridoxal modified enzyme has a characteristic absorption peak at 325 nm indicating the presence of a N^{ϵ}-pyridoxyllysyl derivative. Such a derivative was identified in hydrolysates of the modified enzyme by means of column chromatography. The results obtained with P-pyridoxal-modified enzyme indicate that a lysyl residue is essential for activity by being involved in binding of the cosubstrate NADPH. These results are corroborated by kinetic studies showing competition between P-pyridoxal and NADPH for the binding site. The reactivity of the essential lysyl residue toward P-pyridoxal is significantly increased in the presence of phenol. Inhibition by excess phenol shifts toward lower concentrations in the presence of P-pyridoxal. On the basis of the present results together with previous findings, we propose that phenol acts as a substrate effector by causing a conformation change which exposes a reactive lysyl residue with a concomitant burying of the essential SH groups and a tighter attachment of FAD.

Phenol hydroxylase (EC 1.14.13.7)[1] initiates the inducible metabolic pathway leading from phenol to β-ketoadipate (Neujahr & Gaal, 1978; Gaal & Neujahr, 1979). Although many soil bacteria can be induced to metabolize phenol, attempts to isolate the phenol hydroxylating enzyme from this group of microorganisms have not been successful thus far. We have earlier reported that certain yeasts can be induced to metabolize phenol and that cell-free preparations of such yeasts can hydroxylate phenol in vitro (Neujahr & Varga, 1970; Neujahr et al., 1974). We have subsequently purified and characterized phenol hydroxylase from *Trichosporon cutaneum* (Neujahr & Gaal, 1973, 1975). This enzyme catalyzes the reaction



It has a molecular weight of 148 000 (determined by gel chromatography) and consists of two subunits (76 000 by $NaDodSO_4$[2] electrophoresis). The enzyme contains 16 cysteinyl residues, all of which have to be in the reduced state for maximum activity and for retention of the enzyme's prosthetic group, FAD (Neujahr & Gaal, 1973, 1975). Phenol acts as a substrate effector, enhancing reactivity of the enzyme toward its cosubstrate, NADPH (Neujahr, 1976; Neujahr & Kjellén, 1978).

Apart from the effector function of phenol and the importance of cysteinyl residues, nothing is known about the active site of phenol hydroxylase. We have therefore decided to study the importance of other amino acids. Indirect evidence, obtained during attempts to immobilize the enzyme, indicated that some free amino group(s) may be of importance since the enzyme was readily inhibited by, e.g., aldehydes and cyclic imidocarbonates (Kjellén & Neujahr, 1979). This led us to suggest a possible involvement of lysyl residue(s) in or near the active site (Neujahr, 1976).

We presently report the complete amino acid composition of phenol hydroxylase and the results of experiments to modify lysyl residues of the enzyme. Many of the currently used reagents for modifying amino groups of proteins also react with their sulfhydryl groups, both groups being nucleophiles. Therefore, we had to protect the sensitive SH groups of phenol hydroxylase during modification with several NH_2 reagents. The results of such experiments are treated briefly under Discussion. Using P-pyridoxal, we were able to selectively modify amino groups without affecting the SH groups. These studies are reported in detail. The results suggest a lysyl residue, essential for the binding of the cosubstrate, NADPH. The reactivity of this lysyl residue toward the modifying reagents is significantly increased in the presence of substrate (phenol).

Materials and Methods

Chemicals. Most chemicals were commercial preparations of reagent grade whenever possible. Most of them came from Sigma Chemical Co. (St. Louis, MO), except inorganic salts and phenol which came from Merck (Darmstadt, West Germany). Methyl acetimidate was from Pierce (Rockford, IL), and Blue Dextran and Sephadex products came from Pharmacia Fine Chemicals (Uppsala, Sweden).

Enzyme, Enzyme Assays, and Protein Determinations. The enzyme used was phenol hydroxylase from the yeast *Trichosporon cutaneum*, induced by growth with phenol and prepared essentially as described earlier (Neujahr & Gaal, 1973, 1975). Protein content was either determined by the biuret method with bovine serum albumin as a reference, or

† From the Department of Biochemistry and Biotechnology, The Royal Institute of Technology, S-100 44 Stockholm, Sweden. *Received November 28, 1979; revised manuscript received May 5, 1980.* This work was supported by The Swedish National Research Council, Grants No. NFR B 2427-100 and 2427-102.

[1] Enzyme (EC 1.14.13.7): phenol hydroxylase; phenol, NADPH:oxygen oxidoreductase (2-hydroxylating).

[2] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonate; P-pyridoxal, pyridoxal 5′-phosphate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate; FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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it was computed from the absorption spectrum of the enzyme (Neujahr & Gaal, 1975). The enzyme was routinely assayed by recording oxygen consumption with a Yellow Spring Instrument 4004 Clark oxygen electrode (Yellow Spring Instrument Co., Yellow Spring, OH) in a 2-mL stirred chamber, thermostated at 25 °C, or by following NADPH oxidation in a spectrophotometer (decrease of absorbance at 340 nm). Assays during inhibition studies as well as during modification experiments were always by the oxygen consumption method, and their details are given in the respective legends.

FAD content of the modified enzyme was estimated from its absorbance at 450 nm after separation of excess reagents on a Sephadex G-50 column. Amino acid analyses and identification of N^{ϵ}-pyridoxyllysine were carried out by Dr. David Eaker of The Central Amino Acid Analysis Laboratory, Institute of Biochemistry, Uppsala University, using a Durrum D500 amino acid analyzer. A synthetic tripeptide with carboxy-terminal lysine was pyridoxylated and hydrolyzed, and the hydrolysate was used as a reference.

Free amino groups were determined with TNBS[2] according to Habeeb (1966). Their number was calculated from the absorbance at 335 nm by using an absorbance coefficient of $\epsilon = 1.3 \times 10^4$ M^{-1} cm^{-1} for TNP-amino groups.

Pretreatment of Enzyme. Shortly before each experiment, the enzyme solution was passed through a column (0.9 × 10 cm) of Sephadex G-50, equilibrated with appropriate buffer, to remove dithiothreitol and excess FAD. We routinely add these compounds to protect the enzyme upon storage (Neujahr & Gaal, 1975).

Covalent Modification of Amino Groups. Modification of α-amino groups was done by transamination according to Dixon & Fields (1972). The procedure with P-pyridoxal is described in Figures 2 and 3. Modification with citraconic anhydride was essentially according to Atassi & Habeeb (1972), with cyanate according to Stark (1972) and with methyl acetimidate as described by Hunter & Ludwig (1972). Between 3 and 30 mg of enzyme protein was used. Prior to reactions with citraconic anhydride, TNBS, and cyanate, the enzyme's sensitive SH groups were protected by reversible blocking with sodium tetrathionate or Nbs_2.[2] Controls treated in the same way, excluding only the amino group reagent, were run alongside. After the respective modifications, excess reagents was always removed from the protein on a column of Sephadex G-50 by using conditions as above.

Results

Amino Acid Composition. The enzyme was hydrolyzed in 6 N HCl for 24 and 78 h, and the results were extrapolated to zero time. Total cysteine was determined as cysteic acid after oxidation with performic acid and tryptophan after hydrolysis with β-mercaptoethanesulfonic acid. Table I shows the results. The enzyme contains 1121 residues with a predominance of aspartic and glutamic acids. This is reflected in the acidic character of the enzyme (pI = 5.2). There is, however, a large content of basic amino acid residues, including 70 lysines, 75 arginines, and 38 histidines.

Inhibition by P-pyridoxal. Phenol hydroxylase is inactivated by incubation with P-pyridoxal, at least in the pH range that we have tested (6.5–7.6), i.e., close to its optimum at pH 7.6. The inhibition was studied by preincubating the enzyme with increasing concentrations of P-pyridoxal for varying length of time at 25 °C. In one set of experiments the enzyme was then diluted for immediate assay of catalytic activity. Under such conditions, the inhibition was rapidly (and to a considerable extent) counteracted by dilution of the inhibited enzyme. This indicated a considerable degree of reversibility of the inhibition. In another set of experiments, the substrate and cosubstrate were added directly to the preincubated enzyme and the catalytic activity was recorded. In a third type of experiment the complex between the enzyme and P-pyridoxal was stabilized by reducing the Shiff base with sodium borohydride and separating the modified protein from excess reagents. The catalytic activity of the modified enzyme was then determined and related to the number of P-pyridoxal equivalents that had been attached to the enzyme (Figures 2 and 3). Evidence that P-pyridoxal binds to lysyl residue(s) is provided by the absorption spectrum of the modified enzyme showing a characteristic peak at 325 nm (Figure 3) and by the separation of N^{ϵ}-pyridoxyllysine from hydrolysates of the enzyme modified with P-pyridoxal.

Table I: Amino Acids in Phenol Hydroxylase[a]

	no. of residues/148 000 g of enzyme[b]		
	batch 1	batch 2	accepted
Asp	118.0	118.0	118
Glu	124.8	121.8	123
Lys[c]	71.0	68.6	70
Arg	75.0	74.0	75
His	37.4	37.7	38
Met	22.2	27.1	25
Cys	15.0	16.4	16
Ser	69.9	73.7	72
Thr	65.1	66.8	66
Gly	75.2	77.3	76
Ala	84.3	87.6	86
Val	74.5	72.6	74
Leu	69.7	69.2	70
Ile	72.7	65.6	69
Phe	43.6	42.6	43
Tyr	39.3	39.1	39
Trp	13.7	14.0	14
Pro	45.2	47.9	47
total residues			1121

[a] Conditions: M_r 148 000; two FAD; two subunits; pI = 5.2. Two batches of enzyme were analyzed. They had been purified from two different cultures, induced by growth with phenol as major carbon source. [b] The values refer to molecular weight of the enzyme as determined by gel chromatography and corrected for two FAD but not for hydrogen-bonded water. [c] A third batch of enzyme was modified with P-pyridoxal and hydrolyzed. The hydrolysate was injected into the Durrum D500 column. A new peak, eluting between histidine and lysine, was identified as N^{ϵ}-pyridoxyllysine by comparison with an authentic standard.

The data in Figure 1 illustrate the dramatic effect of P-pyridoxal on enzyme activity. With an ~900-fold molar excess (0.5 mM), half of the catalytic activity is lost within 2 min. The inhibition does not go to completion but seems to stabilize at ~5% of the "zero time" activity.

Stoichiometry of P-pyridoxal Binding. The stoichiometry of the inactivation was determined in two different ways, from the kinetic data (Figure 1) and from the covalent modification experiments (Figure 2). The kinetic method was analogous to that described for the inactivation of myosin by 2,4-dinitrophenol (Levy et al., 1963). Pseudo-first-order rate constants (k') were determined from the time intervals corresponding to 50% inactivation (Figure 1). A log–log plot of k' vs. the concentration of P-pyridoxal is shown in the inset. Its slope is close to 1.0. This indicates that the activity disappears with the binding of 1 mol of P-pyridoxal to 1 mol of enzyme. A very similar result is obtained from the covalent modification experiments shown in Figures 2 and 3. Extrapolating the initial phase of the curve relating disappearance of activity to the number of moles of P-pyridoxal bound (Figure 3) indicates that modifying one lysyl residue is sufficient to inhibit the enzyme. Figure 2 provides evidence that

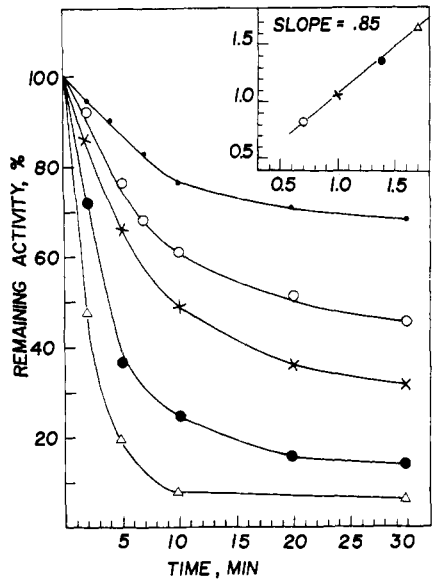

FIGURE 1: Inhibition of phenol hydroxylase by increasing concentrations of P-pyridoxal. Enzyme (87 μg/mL, 0.57 μM) was incubated at 25 °C in 0.1 M Mops–KOH, pH 7.2, with the indicated concentrations of P-pyridoxal. Phenol (to give 0.2 mM) and NADPH (to give 1 mM) were then added in a rapid sequence, and the oxygen consumption was recorded. Uninhibited enzyme had an activity of 1.9 μmol of O_2 min^{-1} (mg of protein)$^{-1}$, which is set as 100%. The activity of controls, incubated under identical conditions without P-pyridoxal, remained unchanged. Symbols in main figure and inset refer to concentrations of P-pyridoxal: (·) 10 μM; (○) 50 μM; (×) 100 μM; (●) 250 μM; (△) 500 μM. The inset shows the relation between log [P-pyridoxal] (M × 10^5) (abscissa) and log [k' × 10^2] (ordinate), where k' denotes the pseudo-first-order rate constant of inactivation. The k' values are determined from time intervals corresponding to 50% remaining activity (Levy et al., 1963).

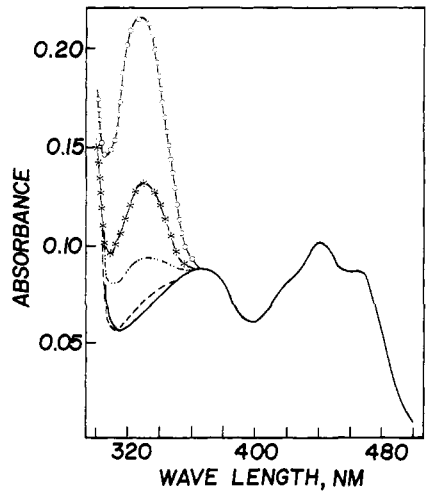

FIGURE 2: Absorption spectrum of phenol hydroxylase before and after modification with P-pyridoxal. Enzyme, 3 mg of protein in 1 mL of 0.1 M Mops–NaOH, pH 6.5, containing a few drops of octyl alcohol to prevent foaming, was mixed with P-pyridoxal, 50 μM–5 mM. After incubation at 0 °C for 10 min, 50 μL of freshly prepared 0.5 M $NaBH_4$ was added in five 10-μL portions during a period of 5 min. After further incubation for 5 min, the reaction mixture was passed through a Sephadex G-50 column equillibrated with Mops–NaOH, pH 7.6. All spectra are normalized to a protein concentration of 1 mg/mL. The formation of N^{ϵ}-pyridoxyllysine derivative is indicated by the peak at 325 nm (Fischer et al., 1963). Unmodified enzyme and all modified preparations in the region 370–500 nm (—). Modified with P-pyridoxal: (---) 0.05 mM; (–·–) 0.5 mM; (*) 1.0 mM; (○) 5.0 mM. Note that the flavoprotein spectrum of phenol hydroxylase is not affected by the modification.

P-pyridoxal is bound to a lysyl residue, the peak at 325 nm indicating the formation of an N^{ϵ}-pyridoxyl derivative (Fischer et al., 1963). The formation of the N^{ϵ}-pyridoxyl derivative of the enzyme does not affect the binding of its prosthetic group, FAD, as indicated by the absence of spectral changes in the 400–500-nm region.

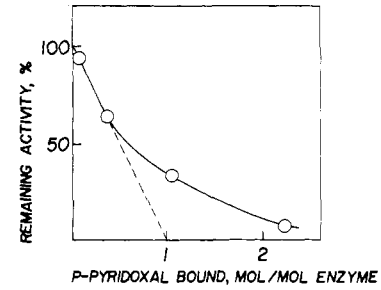

FIGURE 3: Disappearance of enzyme activity upon modification with P-pyridoxal. Enzyme concentration was 0.02 mM. Concentrations of P-pyridoxal from left to right: 0.05, 0.5, 1.0, and 5.0 mM. Experimental conditions are as described in Figure 2. Assay of activity in 0.05 M potassium phosphate, pH 7.6, containing 0.01 mM FAD, 0.25 mM NADPH, and 0.25 mM phenol. The number of P-pyridoxal equivalents that had been attached to the enzyme is deduced from absorbance at 325 nm using ϵ = 9710 M^{-1} cm^{-1} (Fisher et al., 1963; cf. Figure 2). Note that extrapolating the initial phase of the curve indicates total inhibition of enzyme activity with only one modified lysyl residue. 100% activity corresponds to an oxygen consumption of 1.6 μmol of O_2 min^{-1} mg^{-1}. This is equal to the activity of the enzyme before modification and also to that of the $NaBH_4$ treated control.

Table II: Effect of Increasing Concentrations of NADPH and Phenol on the Modification of Phenol Hydroxylase by P-pyridoxal[a]

substrate concn (mM)	P-pyridoxal incorpn (mol/mol of enzyme)	remaining act. (%)
NADPH[b]		
0	1.14	100
0.5	0.93	134
1.0	0.55	202
5.0	0.52	201
phenol[c]		
0	0.85	100
0.05	1.04	48
0.25	1.34	21

[a] Experimental conditions and determination of bound P-pyridoxal as in Figure 2. Assay of activity as in Figure 3. [b] P-pyridoxal concentration = 1.0 mM. 100% remaining activity corresponds to 0.5 μmol of O_2 min^{-1} mg^{-1}. [c] P-pyridoxal concentration = 0.25 mM. 100% remaining activity corresponds to 1.0 μmol of O_2 min^{-1} mg^{-1}.

Effect of Substrate and Cosubstrate on the Inhibition of Phenol Hydroxylase by P-pyridoxal. Also, this problem was studied by both kinetic and covalent modification methods. The results of the modification experiments are shown in Table II. It is seen that increasing concentrations of NADPH during the modification reaction increase the activity of the modified enzyme and decrease the number of incorporated moles of P-pyridoxal. In contrast, the presence during the modification of increasing concentrations of phenol decreases the activity of the modified enzyme and increases the number of attached P-pyridoxal equivalents.

Kinetic experiments were run in the presence of 0, 0.10, and 0.25 mM P-pyridoxal. A double-reciprocal plot of activity vs. NADPH concentration indicates competitive inhibition (Figure 4). A corresponding plot of activity vs. phenol concentration (Figure 5) does not indicate any competition. It seems, instead, that P-pyridoxal causes a shift in the inhibition by excess phenol toward lower concentrations of this substrate.

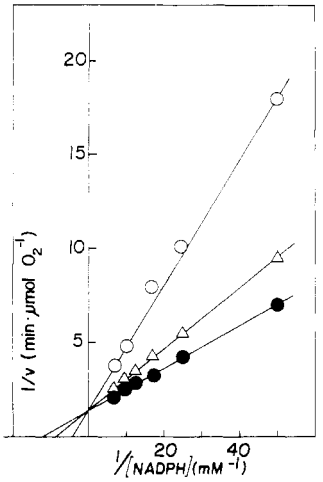

FIGURE 4: Competition between P-pyridoxal and NADPH during catalysis by phenol hydroxylase. Enzyme (62 μg/mL, 0.42 μM with an activity of 1.87 μmol min^{-1} mg^{-1}) was incubated with P-pyridoxal for 5 min using NADPH as the variable substrate with 0.2 mM phenol. Other conditions are as described in Figure 1. (●) No P-pyridoxal; (△) 0.10 mM P-pyridoxal; (○) 0.25 mM P-pyridoxal.

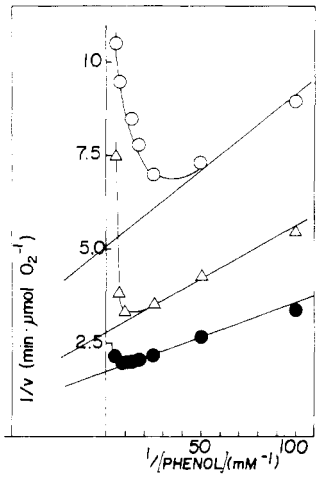

FIGURE 5: Effect of P-pyridoxal on inhibition of phenol hydroxylase by excess phenol. Conditions are as described in Figure 4 with phenol as the variable substrate with 1 mM NADPH. (●) No P-pyridoxal; (△) 0.10 mM P-pyridoxal; (○) 0.50 mM P-pyridoxal.

Effect of P-pyridoxal Analogues, Phosphates, and Nitrogen Bases on Phenol Hydroxylase and on Its Inhibition by P-pyridoxal. Table III compares the effect of P-pyridoxal with that of its analogues. It is seen that P-pyridoxal is by far the most efficient inhibitor. Of the compounds tested, pyridoxal and P-pyridoxamine inhibit slightly at a 1200-fold excess. The low molecular weight aldehydes, formaldehyde, and glutardialdehyde are also inhibitory. It thus appears that the combination of the aldehyde group, the pyridoxine ring, and the phosphate group is important for the inhibitory action of P-pyridoxal on phenol hydroxylase.

The importance of the phosphate group is further illustrated in Table IV. A number of organic phosphates, when present at a 1200-fold excess (5 mM), inhibit the enzyme by 60–70%. Possible competition of such inhibiting phosphates with P-pyridoxal was not evaluated. A significant protective effect is exerted by inorganic phosphate as shown in Table V. Also shown is the protective effect of the nitrogen bases, Tris and cycloserine.

Table III: Effect of P-pyridoxal Analogues on Phenol Hydroxylase[a]

compound	remaining act. (%) after incubation with 0.5 mM (120-fold excess)	5.0 mM (1200-fold excess)
P-pyridoxal	21[b]	5
pyridoxal hydrochloride	97	75[c]
P-pyridoxamine hydrochloride	98	86
pyridoxamine dihydrochloride	104	95
pyridoxine hydrochloride	100	104

[a] Enzyme (62 μg/mL, 0.42 μM) was incubated with the indicated compounds in 0.1 M Mops-KOH, pH 7.2, at 25 °C for 5 min. Phenol (0.2 mM) and NADPH (1 mM) were then added in a mixture, and the rate of oxygen consumption was recorded. The activity in the absence of pyridine derivatives was 1.9 μmol of O_2 min^{-1} (mg of protein)$^{-1}$. Since most of the compounds listed are crystallized as HCl salts, controls with corresponding levels of KCl were included; their activity is set as 100%. This is because the enzyme is inhibited by chloride ions (Neujahr & Gaal, 1973); 0.5 mM KCl leaves 81% and 5.0 mM KCl leaves 67% of activity. [b] A corresponding inhibition of the enzyme (to about 20% remaining activity) is obtained with a 7000-fold excess of formaldehyde or a 1000-fold excess of glutardialdehyde. [c] A corresponding inhibition is obtained with a 1260-fold excess of formaldehyde or a 150-fold excess of glutardialdehyde.

Table IV: Effect of Organic Phosphate Esters on the Activity of Phenol Hydroxylase and on Its Inhibition by P-pyridoxal[a]

compound	concn (mM)	act. (%) after incubation with P-pyridoxal 0 mM	0.1 mM	0.5 mM
none		100	89	20
ATP	5	62		22
ADP	5	67		22
G6P	5	94		23
F6P	5	64		22
F-1,6-P_2	5	64		22
NAD	5	52	59	22
NADP[b]	5	68	56	23
NADP	2.5	77		22
NADP	1.0	100		22

[a] Enzyme (62 μg/mL, 0.42 μM) was incubated with the listed compounds in 0.1 M Mops-KOH, pH 7.2, for 5 min at 25 °C. Phenol (0.2 mM) and NADPH (1.0 mM) were then added in rapid succession, and the rate of oxygen consumption was recorded. The activity of the control was 1.9 μmol of O_2 min^{-1} (mg of protein)$^{-1}$. [b] There is no inhibition by NADP when Mops-KOH buffer is exchanged for potassium phosphate.

Discussion

An Essential Lysyl Residue. The data presented here suggest a reactive lysyl residue in phenol hydroxylase, essential for the binding of NADPH. This conclusion is supported by two lines of evidence. One is the protective action of NADPH against incorporation of P-pyridoxal and against the resulting loss of activity of the modified enzyme (Table II). The other is the kinetically competitive nature of P-pyridoxal inhibition with respect to NADPH (Figure 4). Proof that P-pyridoxal is bound to a lysyl residue is provided by the characteristic spectrum of the *N*-pyridoxal derivative with a peak at 325 nm (Figure 2) and by identification of N^{ϵ}-pyridoxyllysine in hydrolysates of phenol hydroxylase modified with P-pyridoxal. This finding agrees with all the known cases of P-pyridoxal binding to proteins [for a review, see Glazer et al. (1975)]. Lysyl residues are involved in the binding of P-pyridoxal to enzymes requiring this cofactor, e.g., amino acid de-

Table V: Protection of Phenol Hydroxylase against P-pyridoxal Inhibition by Nitrogen Bases and by Inorganic Phosphate[a]

additions	final concn (mM)	P-pyridoxal concn (mM)	act. (%)
			100
		0.25	21
Tris-SO_4	100	0.25	79
		0.50	19
cycloserine[b]	0.5	0.50	26
cycloserine	5.0	0.50	59
cycloserine	2.5	0.25	85
potassium phosphate	5.0	0.25	25
potassium phosphate	25	0.25	29
potassium phosphate	50	0.25	39
potassium phosphate	100	0.25	49

[a] Enzyme (94 μg/mL, 0.63 μM) was incubated with 0.05 M Mops-KOH buffer, pH 7.2, and the indicated additions for 8 min at 25 °C. Phenol (0.2 mM) and NADPH (1.0 mM) were then added in rapid succession, and the rate of oxygen consumption was recorded. Values are given as percent of appropriate controls with activities of 1.6–1.9 μmol of O_2 min^{-1} (mg of protein)$^{-1}$. [b] These assays were in 0.1 M Mops-KOH instead of 0.05 M, with 62 μg of enzyme/mL instead of 94 μg/mL.

carboxylases (Boecker & Snell, 1972). The protection of phenol hydroxylase against P-pyridoxal inhibition exerted by nitrogen bases also supports the idea. To check a possible involvement of α-amino group(s), we treated the enzyme with the "transamination reagent", sodium glyoxylate, and found no effect on activity or FAD content (data not included).

We have also tested a number of other amino-group reagents, among others, TNBS, citraconic anhydride, cyanate, and methyl acetimidate. All these reagents inhibited the enzyme severely. Reversible blocking of the enzyme's sensitive SH groups did not counteract the inhibition. These amino-group reagents caused a more or less extensive and more or less irreversible loss of FAD from the enzyme, indicating profound and largely irreversible conformational changes. The citraconized enzyme, e.g., completely lost the ability to bind FAD. These NH_2 reagents thus introduce a third variable in the modification of phenol hydroxylase—in addition to their effect on NH_2 groups and SH groups (methyl acetimidate is not supposed to react with SH groups, though). Evaluation of the results obtained by using such reagents being uncertain, we do not include their details.

Specificity and Stoichiometry of P-pyridoxal Inhibition. In contrast to the above commonly used NH_2 reagents, the incorporation of P-pyridoxal into the enzyme did not affect binding of its prosthetic group, FAD (Figure 2).

The high specificity of the P-pyridoxal inhibition may depend on a combination of its molecular features, the aldehyde group, the phosphate ester, and the pyridine ring. This is indicated by the data in Tables III and IV. For instance, the magnitude of the inhibition by formaldehyde is comparable to that by pyridoxal at a corresponding molar excess, either compound inhibiting much less than P-pyridoxal (cf. Table III). The inhibition by glutardialdehyde may, in addition, depend on its cross-linking effect. A number of organic phosphate esters inhibit by ~30% when present at a 1200-fold excess (Table IV). On the other hand, inorganic phosphate protects the enzyme toward inhibition by P-pyridoxal (Table V). It is interesting to note in this connection that also the product of the reaction, NADP, inhibits the enzyme, when present at 5 mM concentration in Mops-KOH buffer. There is no inhibition in potassium phosphate buffer, however.

The results of kinetic (Figure 1) and covalent modification experiments (Figure 3) indicate that incorporation of only 1 mol of P-pyridoxal into the enzyme completely abolishes activity. Phenol hydroxylase contains 2 FAD/mol of enzyme composed of two subunits (Neujahr & Gaal, 1973, 1975; Gaal & Neujahr, 1980). One would therefore expect two NADPH to be involved in the catalytic process. The present results thus suggest that the two subunits may not act independently.

Effector Function of Phenol. Phenol increases the incorporation of P-pyridoxal as well as the resulting loss of activity of phenol hydroxylase (Table II). This, together with the evidence of P-pyridoxal binding to a lysyl residue (Figure 2), may indicate that phenol causes a conformational change exposing a reactive lysyl residue. A curious manifestation of this effect is the relation illustrated in Figure 5 showing that in the presence of P-pyridoxal, the excess substrate inhibition by phenol, characteristic of the enzyme (Neujahr & Gaal, 1973), occurs at lower concentrations than in its absence.

The addition of phenol also increases inhibition by most of the other NH_2 reagents that we have tested. Although, as pointed out above, the inhibition by these reagents is extensive, a complete abolishment of activity, e.g., in the case of citraconic anhydride, can be observed only when phenol is present in the reaction mixture. A different effect of phenol was observed with the methyl acetimidate modified enzyme. In this case, phenol protected the enzyme against inactivation, whereas the effect of NADPH could not be evaluated under the conditions of the experiment. Absorption spectra of the enzyme modified without phenol present indicated an extensive loss of FAD. This loss was largely counteracted by the presence of phenol during the modification procedure with a concomitant protection of activity. Thus, the binding of methyl acetimidate to the enzyme does not significantly impair its reactivity toward NADPH. This may be due to the fact that, in contrast to other NH_2 reagents, amidination does not alter the charge of the modified site. Neither is the low molecular size and the absence of aromatic structure from this reagent likely to cause gross conformational changes in proteins (Hunter & Ludwig, 1972). Since amidination requires high pH (higher than pH 8.5), the amino groups that are sensitive to the reagent may be different from those reacting with P-pyridoxal at pH 6.5.

We have previously shown that phenol enhances the reactivity of phenol hydroxylase toward NADPH when measured in the anaerobic reaction (Neujahr & Kjellén, 1978). Phenol also makes the enzyme's SH groups more "buried" (Neujahr & Gaal, 1975). This may result in a tighter attachment of FAD to the enzyme, since its SH groups are somehow involved in the retention of FAD (Neujahr & Gaal, 1973, 1975). From all these results, together with those reported presently, emerges the idea of how phenol acts in its property of effector. It is by causing a conformational change which leads to the exposure of a reactive lysyl residue. At the same time, the enzyme's essential SH groups become more buried with a concomitant tighter attachment of FAD to the enzyme.

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Mitochondrial Hexokinase of Rat Hepatoma Cells in Culture: Solubilization and Kinetic Properties†

Ernesto Bustamante‡ and Peter L. Pedersen*

ABSTRACT: The highly glycolytic hepatoma cell line H-91 is characterized by a high hexokinase activity relative to rat liver; 50% of this activity is associated with the mitochondrial fraction [Bustamante, E., & Pedersen, P. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3735–3739]. Treatment of mitochondria from this cell line with adenosine 5′-triphosphate (ATP) or glucose 6-phosphate solubilizes bound hexokinase activity. Solubilization of the enzyme by ATP results in a six- to sevenfold purification. Free ATP, unchelated by Mg ions, induces the release of the enzyme from the membrane, whereas the MgATP complex is ineffective. Ethylenediaminetetraacetic acid (EDTA) fails to release mitochondrial hexokinase indicating that the enzyme is not attached to the membrane by divalent cations. Energization of mitochondria is not required for ATP to induce solubilization of bound hexokinase. This is evidenced by (a) the ability of the nonhydrolyzable ATP analogue adenylyl imidodiphosphate to solubilize the enzyme, (b) the inability of uncouplers and inhibitors of oxidative phosphorylation to either solubilize or prevent the release of mitochondrial hexokinase, and (c) the inability of atractyloside to solubilize or prevent the release of bound hexokinase. The bound and the ATP-solubilized forms of mitochondrial hexokinase from H-91 hepatoma cells are kinetically different. When membrane bound, the enzyme has a significantly higher apparent affinity (K_m = 0.25 mM) for its substrate MgATP than when solubilized (K_m = 1.2 mM). Free ATP acts as a competitive inhibitor of mitochondrial hexokinase. Both the membrane-bound and the solubilized forms of mitochondrial hexokinase have about the same apparent affinity for glucose (K_m = 56 and 83 μM, respectively). The experiments reported here provide the first description of the properties and the nature of binding of mitochondrial hexokinase from a tumor cell line growing in tissue culture.

Utter et al. (1945) first observed that the hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) activity found in homogenates of rat brain is markedly reduced upon centrifugation. This observation led Crane & Sols (1953) to examine the hexokinase activities of rat tissues, where they found that 50% or more of the total cell hexokinase activity of brain, heart, kidney, and intestinal mucosa is associated with an unidentified 18000*g* sediment. Johnson (1960) was able to identify the particulate enzyme of brain homogenates as mitochondrially bound. Studies of Asc et al. (1955) and Wu & Racker (1959) were among the first to demonstrate that in some cancer cells a large fraction of the total cell hexokinase activity is membrane bound. These observations have now been extended to other systems. For recent reviews, see Wilson & Flegner (1977); Pedersen (1978); also see Rose & Warms (1967); Bustamante & Pedersen (1977); Graziani (1977); Gellerich & Augustin (1977). Perhaps the first really clear demonstration of an association of hexokinase with mitochondria was presented in a now classical paper by Rose & Warms (1967). They also showed that the hexokinase reactant ATP[1] and the product glucose 6-phosphate can specifically

† From the Laboratory for Molecular and Cellular Bioenergetics, Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received April 4, 1980.* This work was supported by a U.S. Public Health Service Grant (CA 10951) from the National Cancer Institute. E.B. was supported by fellowships from the Lilly Research Laboratories, the Du Pont Co., and the Ford Foundation.

‡ Present address: Department of Physiological Sciences, Universidad Cayetano Heredia, Apartado 5045, Lima 100, Peru.

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