we do not have any evidence for the existence of G proteins coupled with IL 1 receptor, but it is also reasonable to speculate that several different types of G proteins are coupled with IL 1 receptor in different cell types. These different G proteins may regulate the subsequent intracellular biochemical events as proposed in other systems (Burch et al., 1986).

In conclusion, although the physiological function of pp 65 has not yet been established, the purified p 65, purified pp 65, and antibody can be used to study the physiological roles of this moiety. Antibody can also be useful for immunocytochemical analysis. With the specific antibody to pp 65 and the protein sequence of pp 65, we should be able to clone the cDNA to determine the entire amino acid sequence of pp 65.

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Thiol- and pH-Modulated Slow Conformational Changes and Cooperativity of Phenol-Binding Sites in Phenol Hydroxylase†

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ABSTRACT: Spectrophotometric titration of phenol hydroxylase (EC 1.14.13.7) with phenol indicated interacting sites for phenol binding. In the absence of added thiol, the cooperativity was positive up to a pH around 8.0 but negative at higher pH values. With added thiol–ethylenediaminetetraacetate, the cooperativity was negative at all investigated pH values. Conversely, a corresponding titration of an enzyme preparation that had been selectively modified in its two most reactive SH groups indicated positive cooperativity at all studied pH values. This selective modification affects the activity of the enzyme to a very minor degree, in contrast to more extensive SH blocking, which displaces flavin adenine dinucleotide with a corresponding loss of activity [Neujahr, H. Y., & Gaal, A. (1975) *Eur. J. Biochem. 58*, 351–357]. The reactivity of SH groups in the enzyme was significantly decreased after turnover. Thiol treatment restored it to that of the native enzyme. Adding phenol prior to reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the assay of phenol hydroxylase gave immediate linearity and higher initial rates than when NADPH was added first. In the absence of added thiol, there was then a shift of the pH optimum. The results indicate slow conformational changes limiting the rate of the overall reaction. The two most reactive SH groups of phenol hydroxylase, though not participating in any obvious redox reactions, are important for these slow conformational changes and for the cooperativity of phenol-binding sites, wherein the anionic S^- forms may be involved (pK_a for cysteine is 8.35).

The prokaryotic flavin-containing aromatic hydroxylases, which employ reduced pyridine nucleotides as electron-donating cosubstrates, undergo extensive changes in conformation upon binding of their phenolic substrates [for a review, see Ballou (1984) and Massey and Hemmerich (1975)]. These changes affect absorption, fluorescence, and circular dichroism spectra. Perturbation of absorption spectra or quenching of flavin fluorescence upon binding of phenolic substrates to these enzymes has been used to measure binding parameters. No

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indications of interacting phenol-binding sites have ever been reported for these prokaryotic enzymes. By contrast, interaction of phenol-binding sites (Neujahr, 1984) has been observed in phenol hydroxylase (EC 1.14.13.7), the first flavin-containing aromatic hydroxylase isolated from a eukaryote, the soil yeast *Trichosporon cutaneum* (Neujahr & Gaal, 1973, 1975). This enzyme is composed of two subunits of apparently equal molecular weight (76K) as determined by sodium dodecyl sulfate ($NaDodSO_4$)[1] electrophoresis. The dimer contains two FAD. The two subunits do not separate upon molecular sieving, ion-exchange HPLC, or electrophoresis. Native phenol hydroxylase contains 16 cysteinyl residues but no disulfide bridge. Reduced SH groups are essential for the retention of the prosthetic group FAD (Neujahr & Gaal, 1975) but have no obvious function in any redox reaction essential for the catalytic cycle (Detmer & Massey, 1984). The present results suggest that the reduced SH groups of phenol hydroxylase, though not participating in any demonstratable redox reactions, are involved in conformational changes during catalysis, participating in the cooperativity of phenol-binding sites, wherein the anionic S^- forms may be involved.

Materials and Methods

All *chemicals* were reagent-grade commercial preparations, whenever available, and purchased as before (Neujahr, 1983). Phenol hydroxylase was induced and isolated from *T. cutaneum* essentially as described earlier (Neujahr & Gaal, 1973) and also by modified procedures (Sejlitz & Neujahr, 1987). Several different preparations were used throughout this investigation. The *enzyme* was routinely assayed by following the phenol-dependent decrease of absorbance at 340 nm. The purified enzyme was stored at −20 or −70 °C for several months or even years without major losses of activity. Before each series of experiments, the enzyme solutions were supplemented with 10^{-3} M dithiothreitol, 10^{-4} M EDTA, and 10^{-5} M FAD and kept for at least 1 h at 4 °C. The excess reagents were then removed by gel permeation chromatography on Sephadex G-25 columns. This treatment usually restored enzyme activity to its original value. Monovalent anions were omitted from all buffers, since they displace FAD from the enzyme. The protein and FAD content of the purified enzyme was determined as before (Neujahr, 1983).

Spectra were recorded in a Cary 219 spectrophotometer under conditions as indicated. Perturbations of the absorption spectra by phenol were used to compute the concentration of enzyme–phenol complexes and of free phenol. These concentrations were then used to construct *Scatchard* and *Hill* plots according to standard textbooks [e.g., see Hammes (1982)].

Selective Modification of the Two Most Reactive Cysteine Residues and Spectrophotometric Titration with Phenol of the Modified Enzyme. The experiment described below is representative of a series carried out under similar conditions but with different amounts of enzyme. Freshly activated enzyme was freed from excess reagents by repeated gel filtration through a Sephadex G25 column equilibrated with potassium phosphate (0.1 M)–EDTA (0.01 M), pH 8.0. The final solution contained 29.4 μM enzyme-bound FAD and was 16.2 μM with respect to the dimeric enzyme (2.4 mg of protein/mL, 1.81 equiv of FAD/dimer). The specific activity was 3.2 units/mg. This solution, 3 mL, was placed in a standard 1-cm cuvette, thermostated at 10 °C, and mixed with 0.11 mL of a freshly prepared 1 mM solution of Nbs_2 in the same buffer (35.4 μM Nbs_2, corresponding to 2.18 equiv of reagent/dimer). The reference cuvette contained a corresponding solution without enzyme. The absorbance at 412 nm was monitored until no further increase could be observed, which took about 3 h, the A_{412} having increased from an initial value of a 0.248 to a final value of 0.743. The $\Delta A_{412} = 0.495$ corresponded to 36.4 μM SH, i.e., 2.39 equiv of SH/dimer, using A_{412} = 13 600 M^{-1} cm^{-1} (Ellman, 1959) or to 35.0 μM, i.e., 2.30 equiv of SH/dimer, using A_{412} = 14 140 M^{-1} cm^{-1} (Gething Davidson, 1972). The solution was passed through a small column of Sephadex G25 equilibrated with 0.1 M BICINE, pH 8.5. Two 3-mL portions of the eluate were used to measure difference spectra during titration with phenol. The FAD content of the modified enzyme was practically unchanged (1.78 equiv of FAD/dimer), whereas the activity has decreased slightly (3.0 units/mg).

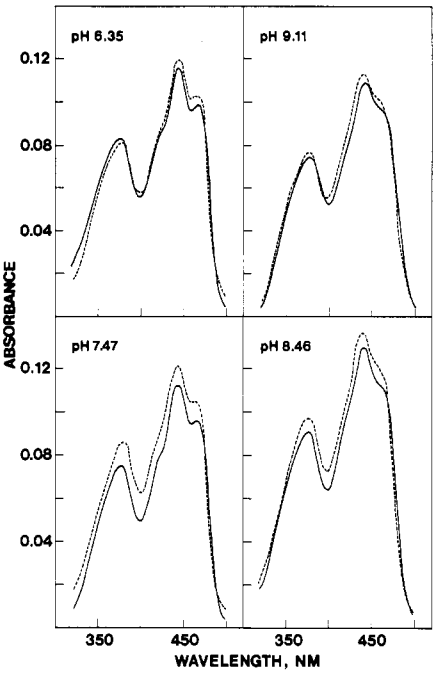

FIGURE 1: Absorption spectra of phenol hydroxylase at 10 °C (6.7 μM enzyme-bound FAD) in the absence (—) and in the presence of 6.7 μM phenol (---). Practically identical perturbation was obtained with higher phenol levels (16.7 and 107 μM phenol, respectively; not shown).

Results

Effect of pH and Thiol–EDTA on the Flavin Absorption Spectrum of Phenol Hydroxylase and on Its Perturbation by Phenol(s). Changes in the absorption spectra of phenol hydroxylase with pH (10 °C), in the absence and in the presence of phenol, are illustrated in Figure 1. At pH 7.5, the spectrum of the enzyme has characteristic shoulders on either side of the maximum at 442 nm, the shoulder around 465 nm being especially pronounced. This shoulder is further accentuated with decreasing pH, whereas it is smoothed out when pH increases. Since the absorption spectrum of free FAD lacks the shoulders occurring in phenol hydroxylase, the observed spectral changes can be assumed to reflect conformational changes in the enzyme affecting the position of the prosthetic group. At all pH values above 7.0, the addition of a few equivalents of phenol increases the absorptivity of the 465–nm

[1] Abbreviations: Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; CHES, 2-(cyclohexylamino)ethanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; HPLC, high-performance liquid chromatography.

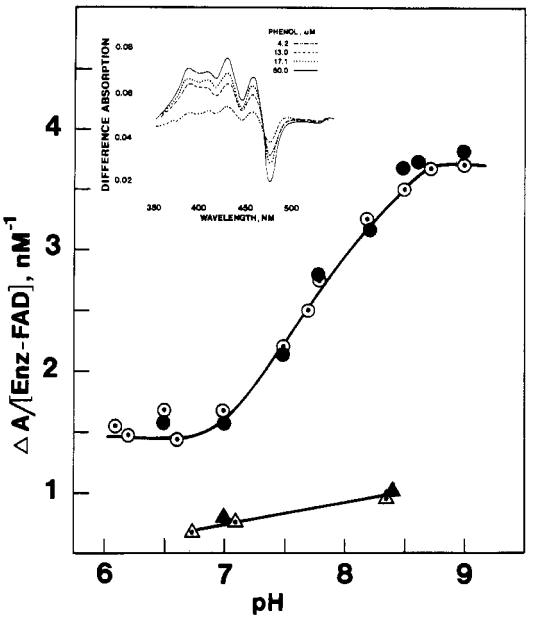

FIGURE 2: pH dependence of the spectral perturbation ($A_{478-458}$) by saturating concentrations of phenol (circles) and resorcinol (triangles). (Closed symbols) Buffer containing 2 mM mercaptoethanol and 0.2 mM EDTA. (Open symbols) Buffer alone. Absorption and difference spectra were taken at 10 °C in 0.05 M buffers using potassium phosphate below pH 8.0 and Tris–sulfate above pH 7.8. The concentration of enzyme–FAD was 8–14 μM. To ensure rapid saturation, 400 μM phenol was used below pH 7.0, while 100 μM phenol was sufficient above pH 7.0. With resorcinol, the saturating concentrations were 100 μM at pH 7.1 and 8.3 but about 400 μM at pH 6.75. The inset shows difference spectra at 10 °C of 24.2 μM enzyme–FAD in 0.05 M potassium phosphate, pH 7.6. Phenol levels are as indicated, the highest level corresponding to maximum difference absorption ($A_{478}-A_{458}$). The reference cuvette contained enzyme–buffer solution without phenol, compensated for dilution.

shoulder and shifts it toward shorter wavelength. This is also the case at pH 6.3, but the part of the spectrum between 300 and 400 nm is somewhat different.

Perturbation of the absorption spectrum of phenol hydroxylase by phenol was used as a measure of phenol binding which was quantitated by means of difference spectra, as illustrated for pH 7.6 in the inset to Figure 2, in the presence of varying concentrations of phenol, up to saturation. The pH dependence of the spectral perturbation between 458 and 478 nm, at saturating phenol concentrations, without or with added thiol and EDTA is shown in Figure 2 (main diagram). As seen, the perturbation increases with pH in the pH range 7.0–8.7, and it is not affected by the presence of added mercaptoethanol–EDTA.

Extrapolation of the perturbation to a saturation value indicated 1.1–1.2 equiv of phenol per enzyme-bound FAD at pH 7.0–8.9, whereas those below pH 7.0 were possibly higher (1.3–3.0), but the values were not very reproducible. There was no appreciable effect of added mercaptoethanol–EDTA on these stoichiometries. A stoichiometry based on two identical, negatively cooperative effector/substrate-binding sites per enzyme dimer was later obtained in the independent experiments using equilibrium dialysis with [^{14}C]phenol (Sejlitz & Neujahr, 1987). Figure 2 (main diagram) also shows that spectral perturbation by resorcinol was much less than that by phenol. As with phenol, the perturbation by resorcinol was not significantly affected by added mercaptoethanol–EDTA. However, extrapolation of the titration values to saturation suggested a stoichiometry that was different from that observed with phenol. In the absence of added mercaptoethanol–EDTA (pH 7.0–8.4), the number of resorcinol

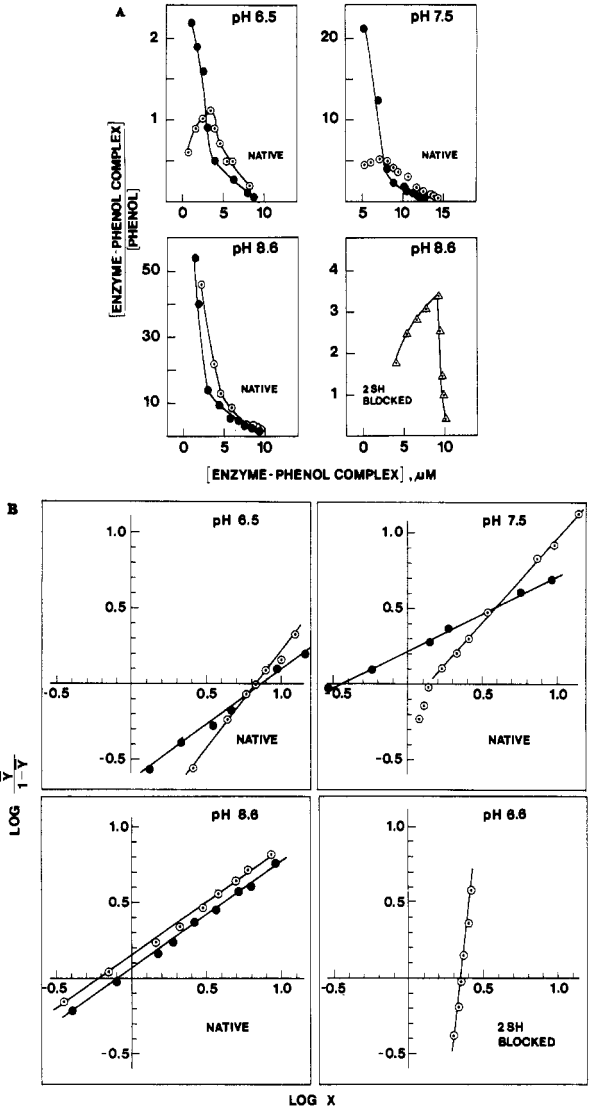

FIGURE 3: (A) Effect of pH and thiol on the profile of saturation curves (Scatchard plots) of enzyme–phenol complexes (10 °C). Note the difference of scales on Y axes. Circles, native enzyme; triangles, enzyme blocked in its two most reactive SH groups. Open symbols, buffer alone; closed symbols, buffer supplemented with 2 mM mercaptoethanol and 0.2 mM EDTA. Buffers: MES for pH 6.5; HEPES for pH 7.5; BICINE for pH 8.5; all adjusted with NaOH. Enzyme concentrations, as enzyme-bound FAD, were 9.1–9.7 μM at pH 6.5, 13–15 μM at pH 7.5, and 12 μM at pH 8.5. (B) Hill plots of phenol binding to phenol hydroxylase at various pHs using native enzyme and enzyme modified in its two most reactive SH groups. Buffers as in (A). Open symbols, buffer alone; closed symbols, buffer supplemented with 2 mM mercaptoethanol and 0.2 mM EDTA.

equivalents varied between 5 and 12 per enzyme-bound FAD. The addition of mercaptoethanol–EDTA at pH 8.4 decreased this stoichiometry to about 2, but the effect at pH below 8.4 was uncertain. The binding of resorcinol and other phenol derivatives was not studied further since this is a side issue in the present context.

Effect of Thiol–EDTA and pH on the Saturation Profiles of Phenol-Binding Sites. Spectrophotometric titrations of phenol hydroxylase with phenol were carried out at several pH values in the pH range 6.5–9.0, in the absence and in the presence of added thiol–EDTA. The perturbations obtained were used to calculate the levels of enzyme–phenol complexes and, by subtraction from the total concentration of phenol, those of free phenol. Figure 3A shows some of the *Scatchard* plots constructed from such data. Figure 3B shows *Hill* plots obtained with corresponding preparations. As seen, the profiles

Table I: pH Dependence of Hill Coefficients[a] for Phenol-Binding Sites in Phenol Hydroxylase As Estimated from Spectral Perturbation by Phenol[b]

pH	Hill coefficient: buffer alone	Hill coefficient: buffer with added thiol–EDTA[c]
6.1	1.2 (1)	nd[d]
6.5	1.2–1.5 (3)	0.6–0.8 (2)
6.7	1.2–1.5 (2)	nd[d]
7.2	1.2–1.3 (2)	0.6 (1)
7.6	1.2–1.6 (5)	0.5–0.7 (3)
8.3	0.8 (1)	0.5 (1)
8.6	0.7 (1)	0.5–0.7 (2)
8.8	0.7–0.8 (2)	nd[d]
9.0	0.6–0.8 (2)	0.6 (1)

[a] Hill coefficient defined according to standard textbooks [e.g., see Hammes (1982)]. [b] Values in parentheses denote number of independent titrations with phenol using four different batches of enzyme. Buffers and amounts of enzyme in the respective experiments were essentially as in Figure 3A,B, 10 °C. [c] Mercaptoethanol (2 mM)–EDTA (0.2 mM). [d] nd, not determined.

of the *Scatchard* plots obtained with the native enzyme in the absence of added thiol–EDTA are convex at pH 6.5 and 7.5 but concave at pH 8.6. However, in the presence of added mercaptoethanol and EDTA, the profiles are concave in the whole pH range that was studied. By contrast, the profile of the plot obtained with the enzyme blocked in its two most reactive SH groups is convex even at pH 8.6.

Hill coefficients deduced from a number of similar experiments with the native enzyme are listed in Table I (cf. Figure 3B). The numerical values of these coefficients varied as indicated. However, they were consistently above 1.0 (1.2–1.6) when computed from experiments in the pH range 6.1–7.6 without added thiol–EDTA, but below 1.0 (0.6–0.8) when computed from those at pH 8.3–9.0. In the presence of added mercaptoethanol–EDTA, these coefficients never exceeded the value of 0.8 in the entire pH range that was studied. These results, together with the curved profiles of *Scatchard* plots (Figure 3A), suggest interacting sites of phenol binding, the cooperativity changing from positive to negative with increasing pH or with added thiol–EDTA.

This observation should be compared to the saturation profile of the enzyme with the two most reactive SH groups blocked. Such a preparation shows positive cooperativity at a pH which favors negative cooperativity in the native enzyme (cf. Figure 3A, Figure 3B, and Table I). The absorption spectrum of this preparation (Figure 4) and its perturbation by phenol indicate a conformation which is quite different from that of the native enzyme.

Effect of Thiol–EDTA and of the Order of Addition of Phenol and NADPH on the Initial Rates of the Overall Reaction. In both the presence and the absence of added thiol–EDTA, equilibrating the enzyme with phenol prior to the addition of NADPH gave immediate linearity and higher initial rates (Figure 5). When NADPH was added first, there was a short lag (1–2 s) before a maximum and linear velocity was obtained. The latter is taken here as the "initial velocity". The effect was more pronounced with no thiol added. In the absence of added thiol, the pH optimum was also affected by the order of addition of phenol and NADPH (Figure 5). When phenol was added first, the optimum was at pH 8.2. When NADPH was added first, the optimum was at pH 7.6. In the presence of added thiol, the optimum was always at pH 7.6 irrespective of the order of addition. Since the perturbation of the spectrum by phenol was complete within the time required to add the ligand and set the spectrophotometer, the present results may suggest slow conformational changes occurring during subsequent steps of the catalytic cycle (e.g., before NADPH binding) and limiting the rate of the overall reaction. This is also indicated by the short but measurable lag in reaching linearity when NADPH is added first (25 °C).

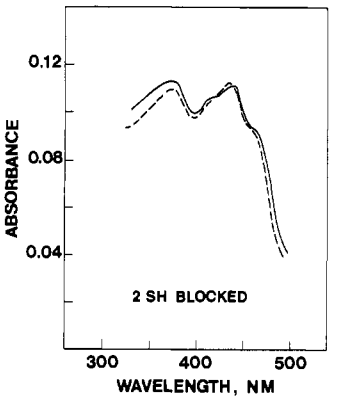

FIGURE 4: Absorption spectrum of phenol hydroxylase (11 μM enzyme-bound FAD) with its two most reactive SH groups blocked (—) and perturbation of this spectrum by 480 μM phenol (---); cf. the spectrum of the native enzyme at pH 8.5 (Figure 1). Maximum perturbation was obtained with about 180 μM phenol. Buffer: 0.1 M BICINE at pH 8.5. Extrapolation of the saturation curve indicated 1.3 equiv of phenol per enzyme-bound FAD.

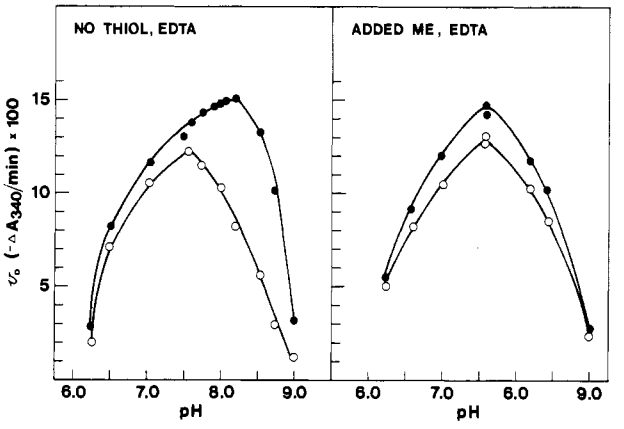

FIGURE 5: Effect of the order of addition of phenol and NADPH on the pH profile of the initial reaction velocity (v_0) of phenol hydroxylase. Phenol added before NADPH (●); NADPH added before phenol (○). Assays were at 25 °C on 20 μg of purified enzyme protein in 3.0 mL of 0.05 M buffers using potassium phosphate for pH 6.0–8.0, Tris–sulfate for pH 8.0–9.0, and sodium pyrophosphate at pH 7.98 and 8.20–8.25. Repeated experiments with Good buffers, using MES for pH 6.0–7.0, HEPES for pH 7.0–8.0, BICINE for pH 7.8–8.8, and CHES for pH 9.0, gave very similar results.

Reactivity of SH Groups after Turnover. Table II shows that the reactivity of the most reactive SH groups, i.e., the initial rate of Nbs_2 reduction, was decreased after turnover. Prolonged storage of the enzyme solution at 4 °C increased this reactivity somewhat. The original reactivity could be restored by treating the enzyme with thiol–EDTA.

Discussion

The values of the *Hill* coefficients or the characteristic deviations from linearity in the *Scatchard* plots are generally accepted as diagnostic tests for cooperativity [cf. Fersht (1984)]. By these tests (figure 3A,B and Table I), the cooperativity of phenol-binding sites in phenol hydroxylase without added thiol–EDTA is positive below pH 8 but negative at higher pH values. However, in the presence of added mercaptoethanol–EDTA, the cooperativity is negative throughout the investigated pH range. The addition of mercaptoethanol–EDTA also abolishes the differences in initial reaction rates and in pH optima caused by the order of ad-

Table II: Reactivity of SH Groups in Phenol Hydroxylase after Turnover[a]

native (N) or during turnover (T)			G25 eluate after turnover, during reaction with Nbs_2			
					SH reacting/dimer (nM μM^{-1} min^{-1}) when tested	
[E–dimer] (μM)	[phenol] (μM)	[NADPH] (μM)	[E–dimer] (μM)	[Nbs_2] (μM)	within 10 min	after storage
			1.4 N	20	1.63	1.68
33.0	830	830	1.4 T	20	0.54	0.54[b]
			0.93 N	17	1.22	1.22
45.8	312	625	1.05 T	17	0.48	0.67[c]
			0.94 N	8.5	1.53	
45.8	78	156	1.06 T	8.5	0.58	
			1.2 N	20	1.53	1.53
28.0	47	78	1.1 T	20	0.58	1.35[d]

[a] Solutions of enzyme, treated as described under Materials and Methods, were mixed with the indicated levels of phenol and NADPH in 0.3–0.6 mL of 0.1 M potassium phosphate, pH 7.6 (no other additions). After 5 min at 22 °C, the solutions were passed through small columns of Sephadex G25 equilibrated with 0.05 M potassium phosphate–0.01 M EDTA, pH 8.0. Portions of the eluates were diluted with the same buffer to 2.94 mL and thermostated at 10 °C in the spectrophotometer. Reference cuvettes contained similar solutions without enzyme. Freshly prepared solutions, 60 μL, of Nbs_2 in 0.05 M potassium phosphate, pH 7.0, were added, first to the reference cuvettes and then to the samples The mixing was completed within 5 s before the recording was started. A_{412} increased rapidly during the first 20 s and then was much slower. The reactivity of the SH groups is calculated from the initial rate of A_{412} increase using $A_{412} = 14\,140\ M^{-1}\ cm^{-1}$ (Gething & Davidson, 1972). E–dimer denotes enzyme dimer (M_r 148K). [b] Four days frozen at 4 °C. [c] Three hours at 4 °C. [d] Three hours with thiol–EDTA at 4 °C, excess reagents removed.

dition of phenol and NADPH (cf. Figure 5). Thus, conditions favoring negative cooperativity, e.g., excess thiol, correlate with those favoring maximum activity of the enzyme. It is tempting to assume that negative cooperativity of phenol-binding sites, which fits into the sequential model (Koshland et al., 1967), is part of the reaction mechanism of phenol hydroxylase whereas the observed cases of positive cooperativity reflect a conformationally less active state of the enzyme. The physiological importance of negative cooperativity may be a possibility to protect the enzyme from toxic effects of phenol derived from nonspecific binding and excess substrate inhibition, which occur already at low phenol concentrations (Neujahr & Kjellén, 1978, Neujahr, 1983). The dissociation constants of enzyme phenol complexes, when calculated from the perturbation of the enzyme's absorption spectrum at 25 °C, in the pH range 7.6–9.2, were below 0.5 μM (not shown). Their values at lower pH were not possible to estimate because the enzyme precipitates during the time required for titration with phenol at 25 °C below pH 7.5. The corresponding dissociation constants at 10 °C decrease from ca. 12 μM to below 1 μM when the pH increases from 6 to 9 (Neujahr, 1984). The decrease is most pronounced above pH 8.3, i.e., under conditions favoring negative cooperativity. This suggests that the anionic form(s) of cysteine(s) may play an essential role in this cooperativity. The pK_a for free cysteine is 8.35; the corresponding values for each of the 16 cysteinyl residues in the enzyme are not known, however. The involvement of SH groups in the slow conformational changes during the overall reaction is also reflected in the significantly decreased reactivity of these groups after turnover (cf. Table II).

Binding of phenol (1-hydroxybenzene) is the first step in the reaction of phenol hydroxylase, which also involves binding of the cosubstrate NADPH and of O_2 to give the final products catechol (1,2-dihydroxybenzene) and H_2O. The enzyme is proposed to follow the same transient and steady-state kinetic mechanism as three other aromatic hydroxylases, viz., salicylate hydroxylase (EC 1.14.13.1) (White-Stevens et al., 1972), *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) (Spector & Massey, 1972), and melitotate hydroxylase (EC 1.14.13.4) (Strickland & Massey, 1973). The established mechanism for this class of three-substrate enzymes is of the type Bi-Uni-Uni-Bi ping-pong (terminology of Cleland (1970)]. In the case of phenol hydroxylase, the mechanism was deduced from steady-state kinetics using resorcinol (1,3-dihydroxybenzene) instead of phenol in order to avoid difficulties derived from substrate inhibition by phenol (Detmer & Massey, 1984). According to the proposed mechanism, a ternary complex is formed by enzyme, resorcinol, and NADPH leading to the reduction of enzyme-bound FAD and a subsequent release of $NADP^+$. Oxygen then adds to the reduced enzyme–resorcinol complex to form a second ternary complex. In this complex, oxygen, while bound to the enzyme flavin, is activated in a series of three transients (intermediates I, II, and III) to perform the electrophilic substitution of the aromatic ring. The cycle is completed by release of 1,2,4-trihydroxybenzene and H_2O. Although steady-state data were consistent with either random or ordered addition of resorcinol and NADPH, a comparison of these data with the respective dissociation constants suggested that binding of resorcinol before NADPH may be the preferred order (Detmer & Massey, 1984). That this is the case with phenol, the principal substrate of phenol hydroxylase, is demonstrated in the present investigation by a different method (Figure 5). The present data show, in addition, that binding of phenol triggers slow conformational changes in the enzyme which affect the intital rate of the overall reaction and that these slow conformational changes are speeded up by adding thiol or by increasing pH, conditions which also influence the cooperativity of phenol-binding sites. As reported elsewhere (Mörtberg & Neujahr, 1987), the kinetics at 25 °C of the overall reaction with all known substrates of phenol hydroxylase are biphasic. With all these substrates, adding phenol before NADPH gives half-saturation constants (the designation "K_m" is not appropriate here because of the biphasic kinetics) that are several times lower than when NADPH is added first. The phenomenon is even more pronounced when the enzyme is measured in situ, in permeabilized cells. It should be mentioned in this connection that in contrast to half-saturation constants at 25 °C those at 10 °C are not significantly affected by the order of addition of phenol and NADPH (M. Mörtberg and H. Y. Neujahr, unpublished results). Thus, as the overall reaction rate slows down, the proposed "slow" conformational changes are no longer rate limiting. All these results support the idea that slow conformational changes and interacting sites of phenol binding form an integral part of the mechanism of phenol hydroxylase and that they may reflect a biological regulatory function.

The cooperative properties of phenol hydroxylase discussed here may be inherent in the dimeric nature of the enzyme. They would thus distinguish this eukaryotic enzyme from the

extensively studied prokaryotic aromatic hydroxylases which are usually described as monomers containing one FAD per single polypeptide chain [e.g., see Higashi et al. (1972) and Husain and Massey (1979)]. However, a recent reassessment of the *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) from *Pseudomonas fluorescens* suggests that this enzyme exists mainly as a dimer (Müller et al., 1979). As in the prokaryotic aromatic hydroxylases, the effector function of the phenolic substrate in phenol hydroxylase is expressed in perturbation of the absorption spectrum (Figures 1 and 2), in quenching of the fluroescence of enzyme-bound flavin (Neujahr, 1983), and in increased affinity toward the reduced pyridine nucleotide cosubstrate (Neujahr & Kjellén, 1978). Phenol hydroxylase undergoes, in addition, a number of changes, triggered by phenol, which are not reported for the prokaryotic aromatic hydroxylases. Thus, in the presence of 1 equiv of phenol, a lysyl residue involved in the binding of NADPH becomes more reactive toward modifying reagents as well as toward NADPH (Neujahr & Kjellén, 1980), whereas the very reactive SH groups of the enzyme become less accessible (Neujahr & Gaal, 1975). At the same time, FAD becomes less prone to be displaced by chaotropic agents (Neujahr, 1983).

Thiol groups and disulfide bridges currently attract much attention in connection with physiologically important refolding processes in, e.g., cell-surface receptors [cf. Craig et al. (1987)]. No disulfide bridges have been found in native phenol hydroxylase (Neujahr & Gaal, 1975). The possibility, however cannot be excluded that such bridges are formed after the enzyme has bound phenol, which makes the reactive SH groups more "buried" (Neujahr & Gaal, 1975) and perhaps more favorably positioned to form such bridges intermittently. Refolding processes would thus be an inherent part of the observed slow conformational changes, which are triggered by phenol and which are reminiscent of those observed in hysteretic enzymes (Frieden, 1979). A meaningful analysis of the hysteretic behavior of phenol hydroxylase, an enzyme which also exhibits allosteric properties (Neujahr, 1984; Sejlitz & Neujahr, 1987), and of the involvement of SH groups in these phenomena will best be done when more is known about its amino acid sequence. This protein contains 1120 amino acids including as many as 16 cysteinyl residues (Neujahr & Gaal, 1975).

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