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Effect of Anions, Chaotropes, and Phenol on the Attachment of Flavin Adenine Dinucleotide to Phenol Hydroxylase†

Halina Y. Neujahr

ABSTRACT: Monovalent anions, ethylene glycol, or Mg^{2+} inhibits the activity of phenol hydroxylase to a degree essentially compatible with the relative degree of their chaotropic power. All these agents affect the spectrum of phenol hydroxylase in a way indicating changes in the hydrophobic interaction between FAD and the enzyme. All agents, except fluoride, abolish the characteristic shoulders on either side of the maximum at 443 nm, bringing the spectrum of phenol hydroxylase closer to that of free FAD. The effect of fluoride is opposite; the shoulders become more accentuated, indicating a more hydrophobic interaction between FAD and the protein than that in the native enzyme. This interpretation is supported by the results of fluorometric measurements. The fluorescence of enzyme-bound FAD is about 10-fold smaller than that of free FAD. In the presence of several monovalent anions the fluorescence of the enzyme increases significantly, whereas in the presence of fluoride it decreases, instead. Displacement of FAD, at 0.015–0.030 M monovalent anions, not giving easily perceptible changes in the primary spectrum, is indicated by difference spectra in the presence of these agents. Absorption spectra of protein eluates from Sephadex G-25 columns, equilibrated with 0.25–1.0 M azide, cyanide, or thiocyanate, indicate complete removal of FAD. The removal of FAD is, to a varying degree, counteracted by low concentrations of phenol. This protective effect of phenol is discussed with view to its known dual function as both effector and substrate of phenol hydroxylase. Spectrophotometric titration of the binding site(s) for phenol reveals one binding site of high affinity ($K_s \simeq 10^{-6}$ M) and additional binding site(s) of much lower affinity ($K_s \simeq 10^{-3}$ M).

Phenol hydroxylase (EC 1.14.13.7)[1] catalyzes the conversion of simple phenols to their *o*-diol derivatives. The enzyme is an FAD-containing monooxygenase with a strict requirement for NADPH. It was originally purified from the soil yeasts *Trichosporon cutaneum* (Neujahr & Gaal, 1973a,b, 1975) and *Candida tropicalis* (Neujahr et al., 1974) after elimination of chloride ions from the purification procedure. Chloride and certain other anions were found to inhibit the purified enzyme (Neujahr & Gaal, 1973a).

The present paper shows that the inhibition reflects displacement of FAD from its proper attachment to the enzyme. This is partly due to chaotropic phenomena. A few equivalents of phenol per enzyme-bound FAD counteracts, to varying degrees, its release. This correlates with various expressions of the dual function of phenol as both effector and substrate of phenol hydroxylase. Some of the results have been reported previously (Neujahr, 1982).

Materials and Methods

Chemicals. All chemicals were reagent-grade commercial preparations whenever available. Most of them were purchased from Sigma Chemical Co. (St. Louis, MO), except inorganic salts and phenol, which came from Merck (Darmstadt, West Germany). Salts of the monovalent anions were of Suprapur quality. Sephadex products were from Pharmacia Fine Chemicals (Uppsala, Sweden). FAD was purified on 1-mm TLC plates in 1-butanol–acetic acid–H_2O (4:3:3) as solvent.

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[1] Abbreviations: phenol hydroxylase, phenol,NADPH:oxygen oxidoreductase (2-hydroxylating) (EC 1.14.13.7); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TLC, thin-layer chromatography.

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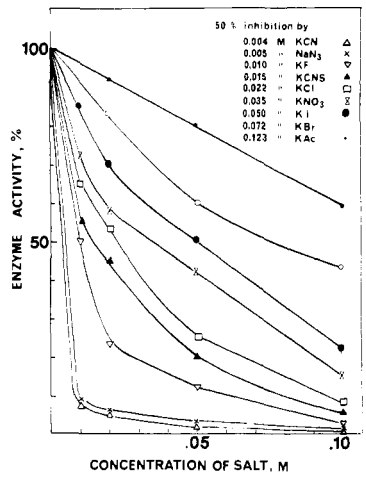

FIGURE 1: Inhibition of phenol hydroxylase by monovalent anions. The uninhibited activity (100%) was 3.2 enzyme units/mg of protein. Enzyme was assayed at 25 C in 3 mL of 0.05 M potassium phosphate, pH 7.5, containing 0.15 M NADPH, 100 μg of purified enzyme protein, and 0.17 mM phenol, initiating the reaction 1 min after mixing with the indicated salts. Selected controls, incubated with salts for longer times, gave identical results.

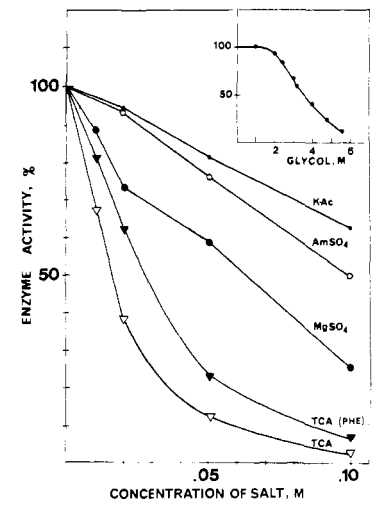

FIGURE 2: Inhibition of phenol hydroxylase by charged and uncharged chaotropes and protective effect of phenol. The uninhibited activity was 3.4 enzyme units/mg of protein. Enzyme was assayed at 25 °C in 2 mL of 0.05 M potassium phosphate, pH 7.5, containing 0.15 mM phenol and 0.15 mM NADPH. If not otherwise stated, the reaction was initiated by the addition of phenol. With trichloroacetate (TCA), an additional set, TCA (PHE), represents assays in which the reaction was started by the addition of NADPH. The inset shows the effect of ethylene glycol, 0–5.6 M (0–35%), with enzyme activity (%) on the ordinate.

Enzyme, Enzyme Assays, and Protein Determinations. Phenol hydroxylase was induced and isolated from *T. cutaneum*, essentially as described earlier (Neujahr & Gaal, 1973b, 1975). The protein content either was determined by the biuret method with bovine serum albumin as a reference or it was computed from the absorption spectrum of the enzyme. The enzyme was routinely assayed by following NADPH oxidation in a spectrophotometer (decrease of absorbance at 340 nm). No significant loss of activity was observed during storage at −18 °C for several months. However, before each series of experiments, the enzyme preparations were "freshened up" by incubation with 10^{-3} M dithiothreitol and 10^{-5} M FAD for 1 h at 4 °C. The excess reagents were then removed by gel permeation chromatography on small (1 × 10 cm) columns of Sephadex G-25. A similar chromatographic procedure was also employed to separate FAD released from the holoenzyme in the indicated experiments.

Spectrophotometry and Fluorometry. Spectra were recorded in a Shimadzu or a Cary 219 spectrophotometer. Fluorescence was measured in a Perkin-Elmer fluorescence spectrophotometer, Model 1000, equipped with a filter on the excitation side and with a monochromator on the emission side. In studies of the effects of salts on the fluorecence of phenol hydroxylase, controls containing free FAD were run in parallel. The values obtained with the enzyme were then corrected for the quenching of free FAD fluorescence by the respective salts.

Results

Effect of Monovalent Anions and Other Chaotropes on the Activity of Phenol Hydroxylase. Figure 1 shows the inhibition of phenol hydroxylase by nine monovalent anions, all but azide supplied as potassium salts. Selected controls with sodium salts gave identical results. The inhibition is most pronounced with cyanide and azide, followed—in decreasing order—by fluoride, thiocyanate, chloride, nitrate, iodide, bromide, and acetate. In Figure 2, the inhibition by acetate is compared to that by the strong chaotropes, the negatively charged trichloroacetate, the positively charged Mg^{2+}, and the uncharged ethylene glycol (inset). Inhibition by ammonium sulfate is included to demonstrate the effect of Mg^{2+} added as magnesium sulfate. The inhibition by trichloroacetate is much larger than that by acetate. It appears from the double set of assays in the presence of trichloroacetate that mixing the enzyme with phenol, prior to the addition of NADPH, offers some protection against the inhibition.

Table I: Fluorescence of Phenol Hydroxylase and Free FAD[a]

solution	fluorescence (arbitrary units)
hydroxylase, 10^{-6} M	605
hydroxylase, 5×10^{-7} M	310
FAD, 10^{-6} M	2800
hydroxylase, 10^{-6} M, + phenol, 4×10^{-6} M	412
FAD, 10^{-6} M, + phenol 4×10^{-6} M	2730

[a] The measurements were in 0.05 M potassium phosphate, pH 7.5 at 11 °C, 5 min after mixing. Excitation was at 364 nm and emission at 520 nm. FAD was purified on 1-mm TLC plates in 1-butanol-acetic acid-H_2O (4:3:3).

Effect of Monovalent Anions and Phenol on the Fluorescence of Phenol Hydroxylase. When FAD is attached to phenol hydroxylase, its fluorescence is quenched almost 10-fold (Table I). The presence of a few equivalents of phenol has a very small effect on the fluorescence of free FAD, but it quenches the fluorescence of the enzyme to about 60%. Several monovalent anions, except fluoride, significantly increase the fluorescence of the enzyme (Figure 3). The most efficient ones are azide and thiocyanate, which increase the fluorescence (values after 15 min) by 800 and 650%, respectively. The effect of fluoride is opposite, giving instead a considerable decrease of fluorescence.

Effect of Chaotropes on the Absorption Spectra of Phenol Hydroxylase. The spectrum of the free enzyme has characteristic shoulders on either side of the maximum at 443 nm (solid line in Figure 4A). The shoulder around 465 nm is especially pronounced. Figure 4C–E shows how this spectrum is affected by the chaotropes thiocyanate, bromide, and ethylene glycol. In all these cases, the shoulder around 465 nm is smoothed out, and the peak at 443 nm is shifted downward and, together with the smoothed-out shoulders, toward longer wavelengths. This makes the spectrum of phenol hydroxylase undistinguishable from that of free FAD (cf. Figure 4B). Similar effects on the spectrum are observed with

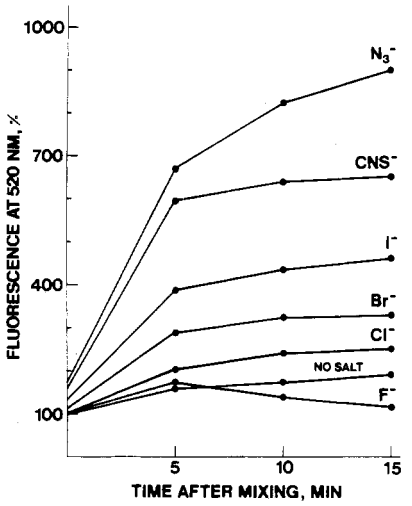

FIGURE 3: Effect of monovalent anions on fluorescence of phenol hydroxylase. Phenol hydroxylase was mixed with the indicated potassium salts; azide was added as sodium salt. The final concentrations were 10^{-6} M enzyme in 0.05 M potassium phosphate, pH 7.6, and 0.05 M salt. Measurements were taken at 11 °C. The values are expressed as percent of fluorescence of phenol hydroxylase in buffer alone at time zero. They are corrected for quenching of the fluorescence of free FAD by the corresponding salts.

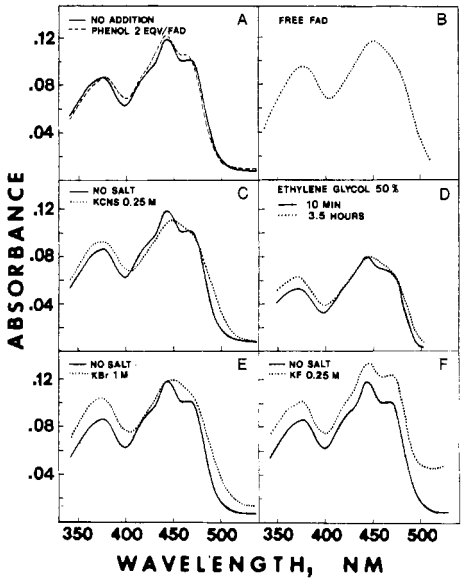

FIGURE 4: Absorption spectra of phenol hydroxylase and free FAD in the presence of various agents. The spectra of the enzyme in the absence of salt, free, and complexed with phenol (A) are shown for comparison. All spectra are in 0.05 M Hepes, pH 7.5 at 25 °C. Potassium phosphate, 0.05 M, pH 7.5, was tested in selected cases. It gave identical results.

azide, cyanide, iodide, chloride, nitrate, acetate, trichloroacetate, and magnesium ion (data not included). The degree of the effect, with equal concentrations of these agents, varies. With most of the small anions, 0.25–1.0 M, the spectrum changes to that of free FAD within 10 min (25 °C); with ethylene glycol, this requires 3–4 h.

The effect of fluoride is entirely different (Figure 4F). There is neither a shift of the peak at 443 nm nor any smoothening of the shoulder around 465 nm. Rather, this shoulder seems to become more accentuated. Higher concentrations of fluoride cause prompt precipitation.

Difference Spectra in the Presence of Thiocyanate and Azide. Concentrations of KCNS, as low as 0.015 and 0.030 M, cause only hardly perceptible changes in the primary spectrum of phenol hydroxylase. However, as seen in Figure

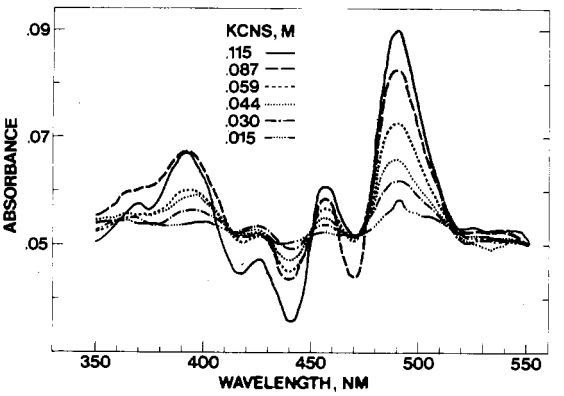

FIGURE 5: Difference spectra of phenol hydroxylase in the presence of KCNS. Enzyme–FAD was at 3.5×10^{-5} M in 0.05 M potassium phosphate, pH 7.5 at 25 °C. Successive levels of KCNS are as indicated. (Reference) Enzyme without KCNS, compensated for dilution.

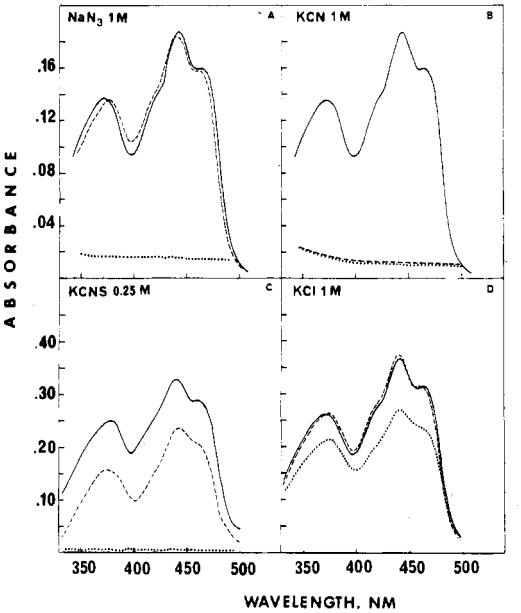

FIGURE 6: Removal of FAD from phenol hydroxylase by monovalent anions and protective effect of phenol. Absorption spectra of protein eluates from Sephadex G-25 columns equilibrated with 0.05 M potassium phosphate, pH 7.5, and salts as indicated, 25 °C (mg of protein/mL of eluate): (A–B) 1.23; (C) 2.23; (D) 2.38. (—) Controls without salt; (···) salt; (– –) salt plus 1 mM phenol.

5, they bring about significant changes in the difference spectrum. These changes may well account for the inhibition shown in Figure 1. The overall pattern of the difference spectrum in the presence of azide (data not included) is similar to that in the presence of thiocyanate (Figure 5). With both agents, there are multiple isosbestic points.

Chaotropic Release of FAD from the Holoenzyme and Effect of Phenol. Figure 6 shows the spectra of phenol hydroxylase after passage through columns of Sephadex G-25, equilibrated with the indicated salts, in the presence and in the absence of added phenol, respectively. Appropriate controls, which have been subjected to nearly identical conditions of gel chromatography in the absence of salt, are included (solid lines). Figure 6 demonstrates that in the presence of azide, cyanide, thiocyanate, and to some extent also chloride, FAD is removed from the enzyme. A protein devoid of FAD appears in the void volume. This release is counteracted by low concentrations of phenol, completely in the case of azide, partly in the case of thiocyanate, and not at all in the case of cyanide.

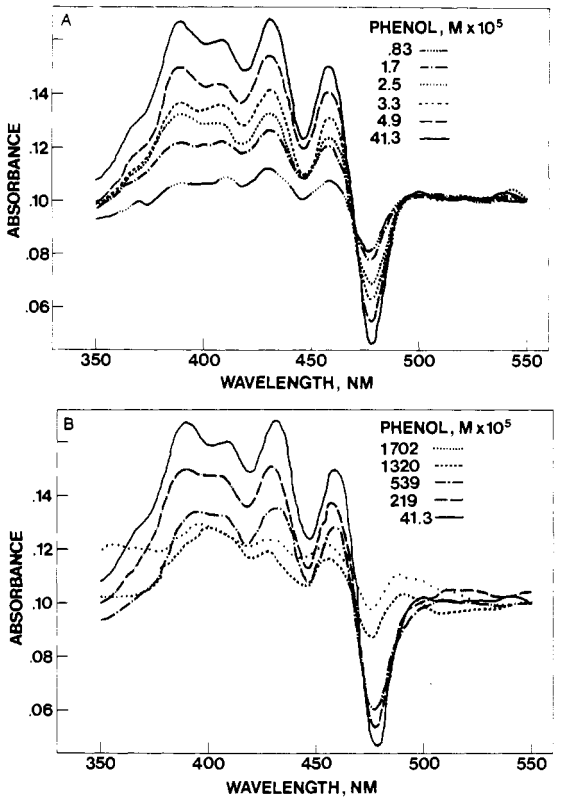

FIGURE 7: Titration of binding site(s) for phenol hydroxylase. Difference spectra were taken at 10 °C. Enzyme–FAD was at 4.76 × 10^{-5} M in 0.05 M potassium phosphate, pH 7.5. Phenol levels were as indicated. (A) Phenol additions until maximum ΔA (478–459 nm), corresponding to 0.41 mM phenol; (B) further phenol additions corresponding to 0.41–17 mM phenol causing successive decrease of the perturbation. (Reference) Enzyme without phenol, compensated for dilution.

Effect of Phenol on Absorption Spectrum of Phenol Hydroxylase. The spectral perturbation by 1–2 equiv of phenol/enzyme-bound FAD is shown by the broken line in Figure 4A. The successive changes in this perturbation with increasing concentrations of phenol are presented by means of difference spectra in Figure 7. The ΔA (478–459 nm) increases until approximately 0.4–0.5 mM phenol has been added (Figure 7). With further successive additions, until 17 mM phenol is present, the spectrum gradually returns to that of the uncomplexed enzyme (Figure 7B).

Discussion

Attachment of FAD to Phenol Hydroxylase and Chaotropic Phenomena. The occurrence of shoulders on both sides of the absorption maximum at 443 nm indicates that FAD in phenol hydroxylase is anchored in a predominantly hydrophobic environment, by analogy to the spectra of free flavins in low polarity solvents (Koziol, 1971). Hydrophobic interactions are associated with an ordered structure of water. They are destroyed by "chaotropic" ions, which facilitate the transfer of apolar groups to water, as indicated by the large positive entropies of their hydrated forms (Hatefi & Hanstein, 1969).

Anions and cations may be arranged in order of increasing chaotropic effects in essentially the following order:

$$PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, I^-, SCN^-, CCl_3COO^-$$

$$NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}$$

According to available literature data, the hydrated forms of SCN^-, I^-, Br^-, and Cl^- carry positive entropies, the one of SCN^- being the largest and that of Cl^- the smallest. In contrast, the hydrated form of F^- carries negative entropy (Hatefi & Hanstein, 1969). This correlates with the effects observed here. Thus, the rapid and efficient removal of FAD from phenol hydroxylase by KNCS (Figures 4 and 6) as well as the strong inhibition by this salt (Figure 1) could be the consequence of the pronounced chaotropic nature of SCN^-. In contrast, the effect of fluoride (Figure 4F), by virtue of its "antichaotropic" nature (negative entropy of its hydrated form), may bring about an even stronger hydrophobic interaction between FAD and the enzyme, as indicated by the deepening of the shoulder aroung 465 nm. Thus, FAD may become attached too tightly to permit the operation of its catalytic cycle, hence, the strong inhibition by fluoride (Figure 1). This interpretation is corroborated by the decrease of fluorescence of phenol hydroxylase in the presence of fluoride (Figure 3).

There are no available literature data on the entropies of hydrated forms of azide and cyanide. However, both ions inhibit phenol hydroxylase even more strongly than thiocyanate (Figure 1). Further, among the anions tested, azide is the most efficient one to increase the fluorescence of phenol hydroxylase (Figure 3).

The multiple isosbestic points in Figure 5 indicate that the displacement of FAD by low concentrations of KCNS is not an "all or none" phenomenon but may proceed through distinct intermediate stages of successive loosening of the FAD–enzyme bonds. Not all such changes give perceptible changes in the primary spectrum. Displacement of FAD seems also to be the cause of the inhibition by trichloroacetate, one of the most potent chaotropic ions available, ethylene glycol, the uncharged chaotrope (Figures 2 and 4D), and Mg^{2+}, the highest chaotrope in the cation series, available as soluble salts. It is interesting to note here the much higher inhibition by trichloroacetate as compared to that by acetate (Figure 2), a much smaller ion that should conceivably penetrate easier into the active site than its halogenated analogue.

The displacement of FAD by small monovalent anions is much more rapid (spectra were taken within 10 min of mixing with the salts) than the displacement by, e.g., ethylene glycol (3.5 h). This indicates that not only the chaotropic nature but also the size and/or the negative charge is of importance for the agent to find its way to the site of its chaotropic action. It is conceivable that small, negatively charged ions can sneak much easier in there, possibly aided by an electrostatic attraction to some positively charged group(s) on the protein, essential to a proper attachment of FAD. Secondary effects that contribute to the inhibition by the small anions cannot, of course, be excluded.

Attachment of FAD and the Effector Function of Phenol. Low concentrations of phenol counteract, to varying degrees, the FAD-displacing effect of the chaotropic anions (Figure 6). This is another manifestation of the effector function of phenol, in addition to those observed earlier (Neujahr & Gaal, 1975; Neujahr & Kjellén, 1978, 1980). Together, they indicate that phenol (a few equivalents per enzyme molecule) causes a conformation change that brings the FAD-binding group(s) deeper inside the catalytic center, makes the SH groups more buried, and promotes the exposure of a reactive lysyl residue essential for binding of NADPH. These effects of phenol actualize the question whether the binding site for phenol in its capacity of effector is the same or different from the binding site in its capacity of substrate. Calculations based on Figure 7 indicate more than one binding site. One binding site has

high affinity for phenol, $K_s(I) = 5 \times 10^{-7}$ to 5×10^{-6} M, and may thus represent the binding site for effector. It is hard to decide whether the second dissociation constant, $K_s(II) = (1-8) \times 10^{-3}$ M, represents just another binding site of a much lower affinity or reflects a more indiscriminate binding of phenol to various regions on the enzyme. Either case may cause a collapse of a specific conformation required in the catalytic cycle, concomitant with return of the spectrum to that of the uncomplexed enzyme (Figure 7B). Both cases would correlate with the phenomenon of excess substrate inhibition observed with phenol hydroxylase (Neujahr & Kjellén, 1978, 1980).

Acknowledgments

I am indebted to Annette Elmblad and Ulla Hägglund for the many fluorometric determinations, only a part of which has been included in the present paper.

Registry No. FAD, 146-14-5; CN^-, 57-12-5; N_3^-, 14343-69-2; F^-, 16984-48-8; SCN^-, 302-04-5; Cl^-, 16887-00-6; NO_3^-, 14797-55-8; I^-, 20461-54-5; Br^-, 24959-67-9; CH_3COO^-, 71-50-1; CCl_3COO^-, 14357-05-2; Mg, 7439-95-4; ethylene glycol, 107-21-1; phenol, 108-95-2; phenol hydroxylase, 37256-84-1.

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Effects of Nucleotides on a Cold Labile Acetyl-CoA Hydrolase from the Supernatant Fraction of Rat Liver†

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ABSTRACT: An acetyl-CoA hydrolase that is labile at low temperature was purified to homogeneity from the supernatant fraction of rat liver. The monomeric molecule, estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, had a molecular weight of about 63 000, while that of the purified enzyme, estimated by gel filtration, was 135 000. Thus, the enzyme consists of two subunits of identical molecular weight. On addition of adenosine 5′-triphosphate (ATP) or adenosine 5′-diphosphate (ADP) at 25 °C, the dimeric form of the enzyme aggregated to tetrameric forms (M_r 242 000 and M_r 230 000, respectively), whereas addition of adenosine 5′-monophosphate had little effect on enzyme association (M_r 145 000). When ATP was removed from the ATP-treated tetrameric enzyme by dialysis, the tetramer was mostly dissociated into the dimeric form. The apparent K_m values for acetyl coenzyme A of the dimeric enzyme and tetrameric enzyme, reconstituted from the former in the presence of 2 mM ATP, were 170 μM and 60 μM, respectively. The purified dimeric enzyme was inactivated by exposure to lower temperature, especially below 10 °C. The various nucleotides tested partially stabilize the dimeric enzyme at low temperature, ATP being the most effective. Sucrose density gradient centrifugation showed that loss of catalytic activity by cold treatment was accompanied by dissociation of the dimer and tetramer into protomer.

Acetyl-CoA hydrolase (EC 3.1.2.1) catalyses the reaction acetyl-CoA + $H_2O \rightleftarrows$ CoA + acetate. This enzyme was first found in pig heart by Gergely et al. (1952) and has subsequently been found in many mammalian tissues (Hepp et al., 1966; Anderson & Erwin, 1971; Quraishi & Cook, 1972; Knowles et al., 1974; Robinson et al., 1976; Matsuda & Yoshida, 1976; Bernson, 1976; Matsuda et al., 1978; Snoswell & Tubbs, 1978; Grigat et al., 1979). In rats, the activity is high in the liver where enzyme is predominantly located in the mitochondria (Knowles et al., 1974; Snoswell & Tubbs, 1978; Grigat et al., 1979).

F. Isohashi and M. F. Utter (unpublished experiments; cf. Prass et al., 1980) first observed activity of ATP[1]-activated acetyl-CoA hydrolase in rat liver homogenates prepared in buffer containing 1.5 M sucrose.[2] This enzyme, which appears to have an extramitochondrial location, had presumably not been detected previously because of its extreme cold lability in the crude homogenate (Prass et al., 1977, 1980) and in excised liver (Isohashi et al., 1981). Its hydrolytic activity in the presence of ATP in rat liver was demonstrable only when liver homogenate was prepared in buffer containing 1.5 M

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[1] Abbreviations: AMP, ADP, and ATP, adenosine 5′-mono-, 5′-di-, and 5′-triphosphates; NAD^+ and NADH, the oxidized and reduced forms of the coenzyme nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, the oxidized and reduced forms of the coenzyme nicotinamide adenine dinucleotide phosphate; PP_i, inorganic pyrophosphate; cAMP, adenosine cyclic 3′,5′-phosphate; ITP, UTP, CTP, GTP, and TTP, inosine, uridine, cytidine, guanosine, and thymidine 5′-triphosphates; PMSF, phenylmethanesulfonyl fluoride; DTT, DL-dithiothreitol; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

[2] Isohashi–Leiter–Utter medium (ILU medium) (Atkin et al., 1979; Murphy et al., 1981) consisting of 50 mM potassium phosphate buffer (pH 7.4), 1.5–2.0 M sucrose, and 0.5 mM EDTA.

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