SOLUBILIZATION OF ACETANILIDE HYDROXYLASE FROM HOG LIVER MICROSOMES

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Very little is known yet about the mechanism of microsomal hydroxylation reactions. This is due to the fact that so far all attempts to solubilize and isolate the enzymes involved have been unsuccessful. Recent studies in our laboratory were devoted to this problem. As a suitable and distinct model reaction for our investigations on microsomal hydroxylations we selected the conversion of acetanilide to p-hydroxy-acetanilide (Mitoma et al., 1956).

A major problem, which frequently is rather difficult to solve, is the solubilization of microsomal enzymes in an active form. Recently, Imai and Sato (1960) in a preliminary note reported the solubilization of an aniline hydroxylating system by treatment of rabbit liver microsomes with snake venom. Indepently from these authors, we were successful in obtaining clear soluble supernatants with acetanilide hydroxylase activity by incubation of hog liver microsomes with purified pancreatic lipase. In this paper we wish to present our results on the solubilization of a microsomal hydroxylating system. In addition, first observations with soluble hydroxylase fractions obtained by ammonium sulfate fractionation of the supernatant are reported.

a) Determination of hydroxylase activity : The fractions to be tested were incubated in 0.1 m tris-phosphate buffer pH 8.2 in the

presence of acetanilide, TPNH or a TPNH-generating system (glucose-6-phosphate, glucose-6-phosphate dehydrogenase and TPN) at a temperature of 37°C. After 3o minutes incubation the reaction was stopped by adding trichloracetic acid. The p-hydroxyacetanilide formed was extraced with isoamylalcohol/ether, reextraced from the organic phase with n/10 NaOH and determined colorimetrically with the reaction of Folin and Ciocalteu (1927).

b) Lipase treatment: The acetanilide hydroxylating activity of (large scale prepared) hog liver microsomes was found to be about 0.20 mμMoles/min./mg protein. This is three times less than the average activity of rat liver microsomes (Krisch and Staudinger, 196o). Hog liver, however, can easily be obtained even in large amounts; furthermore hog liver microsomes are more susceptible to lipase treatment yielding higher activities solubilized than rat liver microsomes. For the solubilization of the hydroxylating enzyme system a concentrated suspension of hog liver microsomes in 0.1 m tris · HCl buffer pH 8.5 (microsomes corresponding to about 3 g liver fresh weight/ml) was incubated for 60 minutes with a purified dialysed lipase preparation (Strittmatter and Velick, 1956; Krisch and Staudinger, 1958). Following this step the microsomal suspension was cooled down to 0-2° and centrifuged for 90 minutes at 140 000 g_{max} in the Spinco ultracentrifuge. The clear red supernatant contained 1o-2o % of total microsomal protein and 15-25 % of the original acetanilide hydroxylating activity. The specific activity in the supernatant was 1.5 to 2.0 times higher than in the original microsomes.

c) Ammonium sulfate fractionation of the supernatant: After having achieved solubilization of acetanilide hydroxylase activity from the microsomes we have made attempts at the further purification of the hydroxylating enzyme system. Following the lipase treatment of large amounts of hog liver microsomes (we usually prepare microsomes from about

1500 g liver) it is convenient to add ammonium sulfate to 40 % saturation prior to centrifugation of the preparation. Thus a better sedimentation of the microsomes non-solubilized by the preceeding lipase treatment is achieved in the Spinco ultracentrifuge (rotor L 30, 90 minutes at 105 000 g_{max}) without any loss of solubilized hydroxylase activity. The clear red supernatant obtained this way was fractionated with ammonium sulfate in four additional steps. The resulting precipitates were centrifuged down and dissolved in about 30 ml 0.1 m tris-phosphate buffer pH 8.2 yielding four clear soluble, faintly reddish protein fractions. Table I shows the specific activities and the cytochrome b_5 content of the various fractions:

Table I Acetanilide hydroxylating activity and cytochrome b_5-content of ammonium sulfate fractions

Fraction	Acetanilide-Hydroxylase (mμMoles/min./mg prot.)	Cytochrome b_5 (mμMoles/mg prot.)
1) Hog liver microsomes	0.21	?
2) Supernatant 40% saturation	0.70	?
3) Fraction 40-50% saturation	0.00	?
4) Fraction 50-60% saturation	0.29	0.25
5) Fraction 60-70% saturation	0.55	0.53
6) Fraction 70-80% saturation	0.16	0.94
7) Supernatant 100% saturation	0.00	?
8) Fraction pH 5.2	0.00	2.91

The highest acetanilide hydroxylating activity was found in the fraction precipitating at 60-70% ammonium sulfate saturation, the specific activity being 2-3 times higher than in the original microsomes. The activity of this fraction, however, was slightly less than the activity of the supernatant 2) obtained at 40% ammonium sulfate saturation. This may be due to a loss of activity during the fractionation procedure or to a separation of a factor essential for full activity. At the present time we cannot decide which of these alternatives is the right one. No

activity could be detected in fraction 3), slight activities were found in fractions 4) and 6). Following saturation with ammonium sulfate we acidified the supernatant with 1 n HCl to pH 5.2. The fraction obtained at pH 5.2 and the cytochrome b_5 precipitating finally at pH 4.2 were inactive with respect to acetanilide hydroxylase activity.

d) Properties of the hydroxylating protein fractions: At first we considered the possibility that a non-enzymatic hydroxylation as described by Udenfriend et al. (1954) might be involved in our experiments. This possibility, however, could be discarded, since boiling of the enzyme preparations for 15 minutes almost completely destroyed the hydroxylase activity.- With different enzyme concentrations a linear relationship was found between protein concentration and the amount of p-hydroxyacetanilide formed.- Dialysis experiments (6 hours at 0-4° against 0.1 m tris phosphate buffer pH 8.2, three times changed) of fraction 5) resulted in no loss of hydroxylating activity.- Spectrophotometric investigations of the protein fractions showed the characteristic absorption bands of cytochrome b_5. The cytochrome b_5 contents of the various fractions as calculated from the molar absorption coefficients given by Strittmatter and Velick (1956) are shown in Table I. The cytochrome b_5 content of two different preparations of the most active fraction 5) was 0.44 respectively 0.53 mμMoles/min./mg protein. However, since the other fractions also contain cytochrome b_5 (though some being inactive with respect to acetanilide hydroxylation) it still remains undecided if cytochrome b_5 is an essential compound of the hydroxylating system or a random contamination of our fractions. Recombination of fraction 5) with purified cytochrome b_5 resulted in no activity increase.- The cytochrome b_5 component of the active fractions could be reduced enzymatically by DPNH of TPNH, indicating the presence of both DPNH- and TPNH-cytochrome b_5 -reductase.

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