BBA 65663

FURTHER STUDIES ON BACTERIAL AND LIVER TRYPTOPHAN PYRROLASES

KEIKO TOKUYAMA*

Cancer Research Institute, New England Deaconess Hospital and Department of Biological Chemistry, Harvard Medical School, Boston, Mass. (U.S.A.)

(Received May 2nd, 1967)
(Revised manuscript received July 26th, 1967)

SUMMARY

Tryptophan pyrrolase was isolated and extensively purified from rat liver and Pseudomonas sp. (ATCC 14675) and the properties of the two preparations were compared. They possess in common the following characteristics: (1) Requirement for a reducing agent to start reaction, (2) Appearance of a lag period in reaction kinetics following preincubation with reducing agent. (3) pH optima in the range 5.5–8.0 (8.0 and 7.0–7.4 for bacterial and liver enzyme respectively). (4) Inhibition by SH reagents and heme- or hematin-chelating reagents added during reaction. (5) Spectral changes in the Soret region during reaction. (6) A mol.wt. of 103 000 measured in sucrose gradients.

The bacterial enzyme is more stable under a variety of conditions than is the liver enzyme; it contains hematin in a more tightly bound form than liver enzyme.

The nature of the prosthetic group and the protein conformation in the active form of the enzyme are discussed.

INTRODUCTION

Tryptophan pyrrolase, the enzyme catalysing the first step in degradation of tryptophan, is induced in both liver and Pseudomonas strains of bacteria by treatment with tryptophan. TANAKA AND KNOX purified the Pseudomonas enzyme and identified it as a hemeprotein[1]. Later, GREENGARD AND FEIGELSON reported that the highly purified liver enzyme was inactive unless hematin was added although their preparation contained material absorbing in the Soret region[2]. KNOX and co-workers[3,4] have described the dissociation and reconstruction of rat-liver enzyme and the effect of some reducing agents on the activation process, and have briefly discussed the reaction mechanisms of both the liver and bacterial enzymes. A reaction mechanism for

Abbreviation: PCMB, *p*-chloromercuribenzoate.

* Present address: Institut de Biologie Physico-Chimique. Paris, France.

bacterial enzyme which differs from that proposed in earlier papers[1,3,4] has been put forward by Feigelson and co-workers[5–8].

In the present study, both enzymes have again been extensively purified in order to clarify and compare in detail their nature and reaction mechanisms.

MATERIALS AND METHODS

Materials

Rats were New England Deaconess Hospital stock (NEDH). Pseudomonas strain RYS-1 (ATCC 14675) originated from Professor R. Y. STANIER's laboratory. Hemoglobin and aldolase were gifts of Professor A. H. MEHLER. Hemin was purchased from Mann Research Laboratories and kept as a stock solution at 0.4 mM in 0.1 M NaOH.

Liver enzyme

Purification of rat-liver enzyme was described elsewhere[3,14] except the $(NH_4)_2SO_4$ fractionation step and the following gel-filtration step (Sephadex G-50). The enzyme prepared in this way was entirely in the apo form as described previously[3]. The holo liver enzyme obtained by addition of hematin to the apo enzyme (usually 0.02 μmoles of hematin per mg protein in the purified preparation) and removal of excess hematin by Sephadex G-50 filtration had an extinction coefficient at 420 mμ of 0.138 per mg protein which remained essentially constant (0.136 per mg protein) after a second Sephadex treatment.

Bacterial enzyme

Conditions for growth and tryptophan induction of Pseudomonas cultures and for extraction and preliminary $(NH_4)_2SO_4$ fractionation of the crude enzyme have been described earlier[1]. Further purification steps were carried out at or below 4°. The $(NH_4)_2SO_4$ precipitate (60% satn.) from 37 g of bacteria (wet wt.) was dissolved in 15 ml of 0.01 M phosphate buffer (pH 7.0) and dialysed overnight against several changes of the same buffer. The dialysed solution 22 ml was passed onto a column (3 cm diameter and 10 cm long.) of DEAE-cellulose previously equilibrated with 100 ml of 0.01 M phosphate buffer (pH 7.0) and the column was eluted batchwise with 0.1, 0.2 and 0.3 M phosphate buffers (pH 7.0). Fractions of 10 ml were collected and assayed. Active fractions were pooled, dialysed overnight against several changes of 0.01 M phosphate buffer (pH 7.0), and the dialysed solution was rechromatographed as before on DEAE-cellulose.

The results of purification of both enzymes are summarized in Table I.

Assay

Enzyme assay and protein measurement were described previously[3,14].

RESULTS

Activation of tryptophan pyrrolase preparations by hematin and reducing agents and dissociation of the prosthetic group from the holo enzyme

In an earlier report, it was shown that the reconstructed holo liver enzyme

TABLE I

PURIFICATION OF LIVER AND PSEUDOMONAS TRYPTOPHAN PYRROLASE

Enzyme	*Purification step*	*Spec. activity (units/mg protein)*	*Apo form (%)*	*Yield (%)*
Liver, hydrocortisone acetate induced	20% liver homogenate supernatant	0.07	—	100
	Crude pH 5 precipitate	0.37	—	93
	Purified pH-5 precipitate before DEAE-cellulose chromatography	0.86	—	83
	DEAE-cellulose eluate	10.0	—	17
	27%–40% satn. $(NH_4)_2SO_4$ fraction	15.8	—	6.3
Bacterial, tryptophan induced	Crude bacterial extract	2.0	—	100
	Dialysed 60% satn. $(NH_4)_2SO_4$ fraction	3.5	20	37
	First DEAE-cellulose eluate			
	0.1 M phosphate fraction	9.3	76 (1st 50 ml) 45 (2nd 50 ml)	
	0.2 M phosphate fraction	16.1	15	28.4
	0.3 M phosphate fraction	17.2	—	
	Second DEAE-cellulose eluate			
	0.1 M phosphate fraction 1st	54.6	92	
	2nd	45.6	38	
	3rd	27.6	25	22.6
	4th	27.2	28	
	0.2 M phosphate fraction	27.6	0	

shows a long lag period before enzyme activity is observable and that this lag is eliminated by the addition at zero time of reducing agents[3]. Similar results have now been obtained with the holo bacterial enzyme (Fig. 1). The results summarized in Fig. 1 show that the Pseudomonas enzyme must have its prosthetic group in the reduced state for enzyme activity to be possible. This confirms similar findings reported earlier for the liver enzyme[1,3,4].

A rapid flow spectrophotometric study of the effect of ascorbic acid on the holo Pseudomonas enzyme activity showed that reaction began within 2 sec after addition of the reducing agent.

Enzyme activity, as is shown above, requires that the prosthetic group of the tryptophan pyrrolase be in the reduced (heme) form. When hematin is reduced by treatment with $NaBH_4$ or ascorbic acid before its addition to the apo liver enzyme, the reaction kinetics of the reconstructed holo enzyme show a definite lag period following addition of substrate[3]. Furthermore, when holo liver enzyme reconstructed from hematin and the apo enzyme is treated with a reducing agent before substrate addition, an initial lag period in reaction kinetics is again observed (Fig. 2). These results suggest that heme has a lower affinity than hematin for apo enzyme.

As reported previously[3] dialysis, Sephadex filtration or acetone extraction of the holo hematin liver enzyme yielded no apo enzyme while the same treatments applied in the presence of ascorbic acid produced some dissociation of the prosthetic group. Further investigation (Table II) has also shown that hematin is not dissociated from the holo hematin liver enzyme but that when reduced to the heme form, it dissociates easily. Dissociation of heme from the holo heme liver enzyme is prevented by the presence of substrate (Table II). Analogous observations have now been made

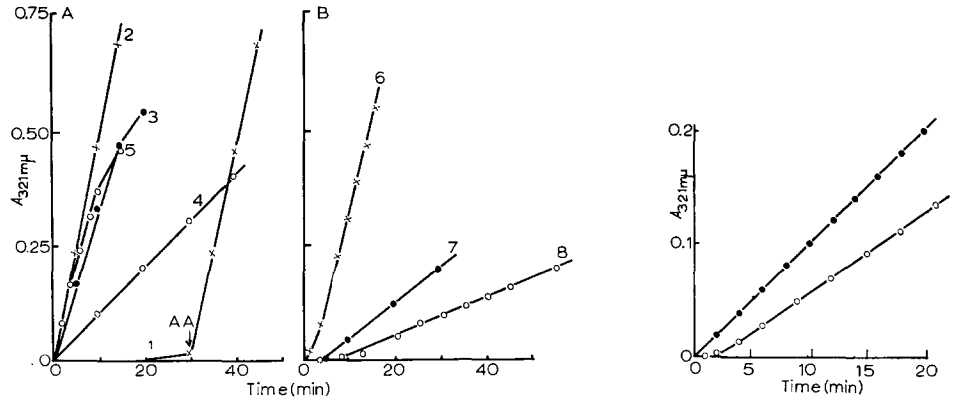

Fig. 1. Effect of reducing agents on the Pseudomonas enzyme catalysed reactions. Assay systems contained in a final vol. of 3 ml, 0.7 ml of 0.2 M phosphate buffer (pH 7.0), 0.2 ml of 0.05 M tryptophan, 0.2 ml of 100% the holo enzyme solution containing 50 μg protein. The following additions were made. A. 1, ascorbic acid (final concn. 0.1 mM) added at $t = 0$; 2, ascorbic acid 0.1 mM added at $t = 30$ (further addition of hematin in 1 or 2 did not modify the enzyme kinetics observed); 3, $NaBH_4$ (5 mM) added at $t = 0$; 4, dithionite (2 mM) added at $t = 0$; 5, dithionite (2 mM) *plus* hematin (1 μM) added at $t = 0$. B. 100% holo enzyme (0.2 ml of enzyme solution containing 250 μg/ml as in A) was incubated with the following additions for 1 min at 25° before addition of substrate. 6, ascorbic acid (concn. during preincubation 3 mM); 7, $NaBH_4$ (concn. during preincubation 15 mM); 8, dithionite (concn. during preincubation 6 mM).

Fig. 2. Delayed reaction produced by preincubation of the liver enzyme with ascorbic acid. 100% holo enzyme was prepared from apo enzyme (EXPERIMENTAL, 1st Section). Assay mixtures were as described for Fig. 1 and contained 40 μg of enzyme protein: 1, ascorbic acid (1 mM added at $t = 0$; 2, enzyme preincubated with ascorbic acid (1 mM) for 2 min at 25° in 0.2 M phosphate buffer (pH 7) before addition of substrate.

with Pseudomonas enzyme (Fig. 1B). Treatment of the holo hematin Pseudomonas enzyme with reducing agents in the absence of substrate produces a lag period in the reaction kinetics observed after subsequent addition of substrate. The duration of the lag period seems to depend on the reducing power (redox potential) of the reducing agent used (dithionite > $NaBH_4$ > ascorbic acid).

Stability of tryptophan pyrrolase preparations and susceptibility for SH reagents

Environmental factors, pH, ionic strength and protein concentration influence profoundly the stability of the purified enzyme during storage. The purified liver enzyme is unstable at both acid and alkaline pH, the latter being the more unfavourable. At neutrality, low ionic strength, media, and low protein concentrations cause large loss of activity during storage, but if either of these parameters is raised, the enzyme is relatively stable. The apo liver enzyme is much more susceptible to environment than the holo enzyme. For example, after storage at pH 7 for 1 and 4 days holo enzyme retained 85 and 63% of its initial activity and the apo enzyme retained only 35 and 19% respectively. It was also observed that the apo enzyme is protected against deactivation by the presence of hematin or tryptophan and that the addition of hematin to enzyme preparations has less protective effect at alkaline than at neutral pH. Addition of tryptophan to a Pseudomonas-enzyme preparation which contained only holo enzyme had an activating effect.

TABLE II

SEPARATION OF THE PROSTHETIC GROUP FROM THE LIVER ENZYME BY SEPHADEX G-50 TREATMENT UNDER VARIOUS CONDITIONS

Enzyme preparations after DEAE-cellulose chromatography and Sephadex G-50 filtration in 0.1 M phosophate buffer (pH 7.0) were treated with excess hematin, tryptophan, and reducing agents for 2 min at 25° in 0.1 M phosphate buffer (pH 7.0). Solutions were then filtered through Sephadex G-50 once or twice, to remove the reagents. The percentage of apo enzyme in the filtrates were measured as described in the legend to Fig. 4. After treatment with reducing agents alone, the apo content was also measured by the decrease in heme absorption at 408 mμ which gave values (given in parenthesis) including denatured apo enzyme. Activity losses during the listed treatments were quantitatively restored by assay in the presence of excess hematin, except where treatment was by reducing agents alone. In this case, the loss of activity was 20–60%.

Expt. No.	*Percentage of apo enzyme*					
	Untreated starting material	*Additions to starting material before final Sephadex G-50 filtration*				
		None	*Hematin (1 μM)*	*Hematin (1 μM); tryptophan (1 mM)*	*Hematin (1 μM); tryptophan (1 mM); dithionite (10 mM)*	*Ascorbic acid (10 mM)*
1	40	40	0	—	—	—
2	50	50	0	—	—	55 (70)
3	78	—	0	0	—	32 (50)
4	80	—	0	0	0*–29 (37)** 40 (56)***	—

* Assayed after treatment but before Sephadex filtration.
** Assayed after one Sephadex filtration step.
*** Assayed after two Sephadex filtration steps.

The addition of ascorbic acid to the enzyme during storage causes rapid loss of activity of both the liver and bacterial enzyme, particularly the former. (With 1 mM of ascorbic acid, no activity was observed in liver enzyme after 1 day's storage, and only 30% of normal bacterial-enzyme activity.) Glutathione had a similar effect on the Pseudomonas enzyme and caused 85% loss of activity after 2 days storage when added at 1 mM. Deactivation by these reducing agents may possibly be due to apo-enzyme formation following reduction of hematin to heme which, as mentioned above, is easily dissociated from the protein moiety of the holo enzyme.

When the reaction with a stored, partially deactivated, enzyme is followed over long periods of time (Fig. 3), recovery of part of the lost activity is sometimes observed. This indicates that purified liver enzyme may be reversibly denatured during storage and renatured in the presence of hematin and substrate. It may be noted that the initial reaction velocity with the liver enzyme stored under unfavourable conditions is similar to that observed (Fig. 3, Curve 4) with the holo-enzyme fraction of the original preparation (*i.e.* without addition of hematin to convert the apo fraction to the holo enzyme). This suggests that it is the apo liver enzyme fraction of the stored preparation which is being reactivated.

Purified liver enzyme is strongly inhibited by *p*-chloromercuribenzoate (PCMB) or monoiodoacetate. Preincubation of the liver enzyme with 20 μM PCMB caused 50% reduction of v_i after 5-min and 84% reduction after 80-min treatment. Addition of the same concentration of PCMB to a reaction mixture during reaction caused an

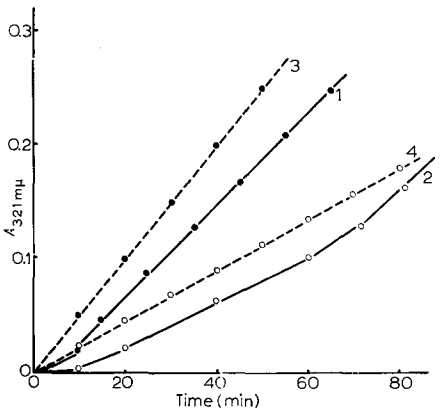

Fig. 3. Reversible denaturation of the liver enzyme. Assay mixtures as described in the legend to Fig. 1 contained: 4, ascorbic acid (final concn. 0.7 mM) added at $t = 0$; 3, ascorbic acid as in (4) *plus* hematin (final concn. 0.7 mM) added at $t = 0$; 1, the enzyme (460 μg protein per ml) aged for 70 h at 4° in 0.06 M phosphate buffer (pH 7.0) ascorbic acid and hematin as in (3); 2, the enenzyme (230 μg protein per ml) aged for 70 h at 40° in 0.01 M phosphate buffer (pH 7.0) ascorbic acid and hematin as in (3).

immediate fall in reaction rate of 50%. In both cases, the degree of inhibition increased during the course of reaction in the presence of PCMB. Higher concentrations of PCMB (0.1 mM) completely inhibit the enzyme. Preincubation of the enzyme with 1.7 mM monoiodoacetate for 5 min causes 43% reduction of v_i, but this inhibition does not increase during subsequent reaction.

The bacterial enzyme, unlike the liver enzyme, is not subject to inhibition by preincubation with SH reagents although a short lag period is observed in the reaction kinetics of the bacterial enzyme preincubated with PCMB. However, if it is preincubated with PCMB in the presence of ascorbic acid, marked inhibition is observed (60% reduction in v_i after 30 min, and complete loss of activity after overnight preincubation). Since PCMB inhibits the enzyme only in the presence of ascorbic acid which, as mentioned above, reduces hematin to the heme form and therefore favours dissociation of the holo to the apo enzyme, it seems possible that the hematin bound to the bacterial enzyme protects a PCMB sensitive site in the enzyme protein.

The results of studies of PCMB inhibition of the Pseudomonas enzyme are summarized in Table III.

Inhibition of tryptophan pyrrolase by hematin-, and heme-chelating agents

A series of agents known as inhibitors of hematin or heme reactivity were tested with the liver and bacterial preparations of the enzyme. The hematin inhibitors, KCN and NaN_3, displayed similar effects. When added to the enzyme before the reaction was started by addition of substrate and ascorbic acid complete loss of activity was caused. However, when added to incubation mixtures in which the reaction was in progress, these reagents initially caused an slight inhibition which became complete 10–20 min later (Fig. 4).

The heme inhibitor CO stops the reaction immediately and completely when added to the enzyme during reaction but inhibits neither liver nor bacterial enzyme

TABLE III

INHIBITION OF PURIFIED PSEUDOMONAS ENZYME BY PREINCUBATION WITH PCMB AND/OR ASCORBIC ACID

The purified enzyme (100% holo form) was incubated with PCMB and/or ascorbic acid, as indicated, at 25° in 0.1 M phosphate buffer (pH 7.0) and residual activity assayed. Values express percentage of initial activity remaining after incubation.

Length of preincubation	*Additions to incubation medium*			
	None	*PCMB (0.7 mM)*	*Ascorbic acid (0.7 mM)*	*PCMB (0.7 mM) + ascorbic acid (0.7 mM)*
30 min	100	90	90*	37
20 h	100	50	50	0

* The enzyme aged for a week became more susceptible (25% residual activity after 30 min preincubation) than the freshly prepared enzyme.

if added to them before the reaction is initiated. Inhibition by CO added during the reaction is competitively overcome by added O_2 (Fig. 4). Both forms of the enzyme gave similar results with the inhibitors just discussed, but behaved differently with *o*-phenanthroline. This reagent stopped the reaction of the liver enzyme, immediately if added to the enzyme when the reaction was in progress, and very rapidly if preincubated with the enzyme before the substrate and ascorbic acid addition. However, with the bacterial enzyme, inhibition developed slowly whether the reagent was added before or during the reaction.

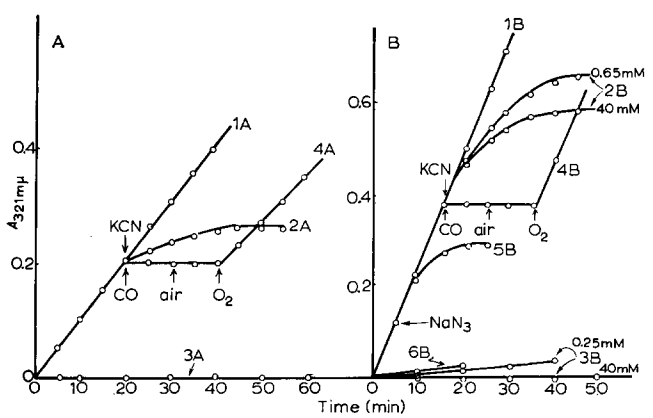

Fig. 4. Inhibition of the liver and bacterial enzyme by heme chelating agents. Standard assay mixtures were used containing 50% holo liver enzyme (40 μg of protein) *plus* hematin at a final concn. of 0.7 μM (Fig. 4A) or 100% holo bacterial enzyme (20 μg of protein) without added hematin (Fig. 4B). The following additions were made: 1A, 1B, none; 2A, KCN, 0.33 mM final concn. at the time shown; 2B, KCN, 40 mM or 0.65 mM (0.25 mM in the case of the preincubation 3B) final concn. at the time shown; 3A, 3B, KCN at the final concns. in 2A, and 2B respectively was preincubated with the enzyme for 1 min at 25° in 0.1 M phosphate buffer (pH 7.0) before addition of tryptophan and ascorbic acid; 4A, 4B, CO was added to incubation mixtures at the times shown and when reaction stopped, air and then O_2 were introduced as indicated; 5B, NaN_3 20 mM final concn. was added at the time shown; 6B, NaN_3 as in 5B, was preincubated with the enzyme for 1 min at 25° in 0.1 M phosphate buffer (pH 7.0) before addition of tryptophan and ascorbic acid.

TABLE IV

THE SORET BAND WAVELENGTHS OF THE DIFFERENT FORMS OF PSEUDOMONAS AND LIVER TRYPTOPHAN PYRROLASE

Enzyme form	*Absorption maximum (mμ)*	
	Pseudomonas enzyme	*Liver enzyme*
EFe^{3+} (Hematin)	404	410
EFe^{2+} (Heme)	430	425
$EFe^{2+} \cdot CO$	420	420
$EFe^{2+} \cdot O_2$	approx. 406	approx. 412

Spectral characteristics of the liver and Pseudomonas enzyme

The Soret band wavelengths of the different forms of the liver enzyme and the bacterial enzyme are shown in Table IV.

Under anaerobic conditions in the presence of excess reducing agent such as ascorbic acid or dithionite, the hematin enzyme peak at 404 or 410 mμ is changed directly into the heme enzyme peak at 430 or 425 mμ respectively. When CO is added to either the bacterial or liver enzyme in the heme form the strongly absorbing peak at 420 mμ characteristic of the form $EFe^{2+} \cdot CO$ rapidly appears.

A study of spectral changes during reaction catalysed by the holo bacterial enzyme under aerobic conditions gave the following results (Fig. 5). Initiation of the reaction by addition of tryptophan and ascorbic acid caused a considerable reduction in the absorption maximum and a slight shift (404→405–406 mμ) to a longer wavelength. As reaction proceeded, the absorption at 405–406 mμ fell further and a shoulder appeared progressively at about 430 mμ. Absorption changes at 405–406 and 430 mμ stopped when the reaction reached equilibrium. It may be noted that the spectra of holo tryptophan pyrrolase (hematin containing) alone (Curve 1, Fig. 5) and *plus* tryptophan (Curve 2, Fig. 5) do not pass through the isosbestic point of the family of spectra obtained during reaction (Curves 3–8, Fig. 5). These observations suggest that in aerobic conditions, the enzyme may be present during reaction as the oxygenated heme form ($EFe^{2+} \cdot O_2$). When the reaction was carried out under semi-anaerobic conditions (partial vacuum), the enzyme was reduced relatively rapidly and the hematin peak finally disappeared (Fig. 6A). Results similar to those described above for the holo Pseudomonas enzyme were obtained in analogous experiments with the holo liver enzyme (Fig. 6B), under partially anaerobic conditions. A reduction in absorption at 410 mμ and a slight shift of the maximum (410→412–415 mμ) were observed together with the appearance of a shoulder at 425 mμ. In aerobic conditions, these changes were obscured by a rapid increase in absorption in the 400–430 mμ region, due to the reaction product. Reduction of the holo bacterial enzyme by tryptophan alone was examined and found to be very slow, even under strictly anaerobic conditions. This finding, similar to a previously reported observation[1] suggests that a mechanism of reaction of tryptophan pyrrolase involving reduction of the enzyme by its substrate is unlikely.

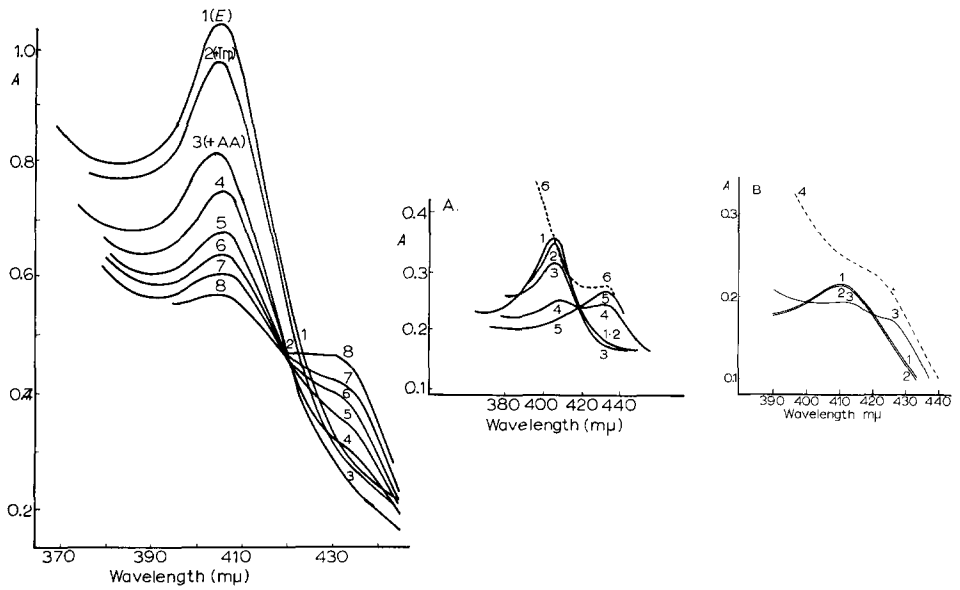

Fig. 5. Changes in the spectrum of the bacterial enzyme during reaction under aerobic conditions. 100% holo enzyme (3.5 mg protein per ml; spec. activity 20) obtained by reconstruction was used. Spectra in the 380–440 mμ region were determined under the following conditions: 1, enzyme alone in 0.05 M phosphate buffer (pH 7.0, vol. 0.95 ml) under aerobic conditions (open to air); 2, after addition of tryptophan (final concn. 5 mM; final total vol. 0.98 ml); 3, after addition of ascorbic acid (AA) to (2) (final concn. 0.5 mM; final vol. 1.0 ml); 4, 5, 6, 7, 8: 5, 10, 15, 20 and 30 min, later, respectively.

Fig. 6. Changes in the spectra of the bacterial and liver enzyme during reaction under anaerobic, and semi-anaerobic conditions respectively. A. Reconstructed 100% holo bacterial enzyme (1.3 mg protein per ml; spec. activity 25) was used. Spectra were determined in the 370–460 mμ region under the following conditions: 1, enzyme alone in 0.05 M phosphate buffer (pH 7) (vol. 3 ml) under anaerobic conditions obtained by evacuation using an oil pump; 2, immediately after addition to (1) of tryptophan (final concn. 5 mM); 3, immediately after addition to (2) of ascorbic acid (final concn. 0.5 mM); 4, 5, 40 min and 60 min after addition of ascorbic acid; 6, after introduction of O_2 into (5). B. Reconstructed 100% holo liver enzyme (0.6 mg protein per ml; spec. activity 12.5) was used. Spectra were measured in the 390–440 mμ region under the following conditions: 1, enzyme alone in 0.05 M phosphate buffer (pH 7.0, vol. 3 ml) under semi-anaerobic conditions obtained by evacuation with a water aspirator (about 30–40 mm Hg); 2, immediately after addition to (1) of tryptophan (final concn. 5 mM) *plus* ascorbic acid (final concn. 0.5 mM); 3, 5 min after addition of tryptophan and ascorbic acid; 4, after introduction of oxygen to (3) and shaking.

Addition of CO to reaction mixtures during the reaction caused the disappearance of the characteristic bacterial enzyme bands (406 and 430 mμ) or liver enzyme bands (412 and 425 mμ) and the appearance of the 420-mμ band shown by the enzyme CO complex. A similar change was produced by addition of CO to the reduced form of either enzyme under anaerobic conditions.

Molecular weights of tryptophan pyrrolase preparations

Purified preparations of both enzymes were centrifuged on 4 ml linear sucrose gradient prepared from 5% and 20% solutions of sucrose in 0.5 M Tris (pH 7.0) using the Spinco SW39 rotor (38 000 rev./min, 16 h, 2°). Hemoglobin (mol. wt. 66 000) and

aldolase (mol. wt. 147 000) were used as markers. Fractions were assayed for hemoglobin content by light absorption measurements and for aldolase and tryptophan pyrrolase content by enzyme-activity determination. Aldolase was assayed according to the procedure of WARBURG AND CHRISTIAN[12]. The liver-enzyme activity could not be observed immedialtely after fractionation of gradients but was measurabe 24 h later. The bacterial enzyme activity could be assayed immediately after centrifugation. A value of 103 350 was found as the mol. wt. of both enzymes.

DISCUSSION

A comparison of the properties of the liver and bacterial enzymes described in the preceding sections shows that enzymes prepared from bacterial and animal sources resemble each other closely in many respects. They possess identical molecular weights, as measured by sucrose-gradient centrifugation, display similar spectral changes during reaction, and are inhibited similarly by SH–reagents and heme- or hematin-chelating agents (with the exception of *o*-phenanthroline; see below) added during reaction. Both enzymes require the presence of a reducing agent in order to start the reaction and both show an initial lag in reaction kinetics if they are pretreated in the holo form with the reducing agent.

Nevertheless the enzymes do not appear to be identical. Thus, while the liver enzyme was obtained, as previously described[3], as a pure apo form after the first DEAE-cellulose chromatography step in the purification process, the bacterial enzyme was never obtained completely in the apo form. During purification of the bacterial enzyme, the proportion of apo enzyme rose from 20% in the $(NH_4)_2SO_4$ fraction to 76% and finally 92% in the earliest fractions of the first and second DEAE-cellulose–0.1 M phosphate eluates respectively. This implies that the holo bacterial enzyme can also dissociate into hematin and the apo form. These results suggest that hematin bonding to the bacterial enzyme is more stable than that bonding to the liver enzymes. This conclusion may be supported by another observation that the liver enzyme is more sensitive to preincubation with PCMB under non-reducing conditions than is bacterial enzyme (Table IV). It seems possible that hematin dissociation is required to uncover a PCMB-sensitive site and in fact reducing conditions which convert hematin to the less strongly bound heme suppress the difference in sensitivity to PCMB shown by the two enzymes.

Studies of the stability of the bacterial and liver enzyme under various conditions (RESULTS, 2nd Section) reveal that the bacterial enzyme is more stable than the liver enzyme. Since the apo liver enzyme is more sensitive to environmental factors than the hole enzyme and more readily formed from the holo enzyme than is apo bacterial enzyme from holo bacterial enzyme, the difference in stabilty of the two enzymes may be related to the dissociability of their prosthetic groups.

The nature of the active form of the enzyme can be considered from two points of view: first, the state of the prosthetic group and, second, the conformation of the enzyme protein. Several observations suggest that in the active form of the enzyme, the prosthetic group is in the heme state. Reaction initiation requires the presence of a reducing agent (RESULTS, 1st Section). Hematin inhibitors, such as KCN and NaN_3 inhibit reaction immediately and completely if preincubated with the enzyme, but cause only progressive though finally complete inhibition when added during reaction,

while the heme inhibitor CO inhibits only when added during the reaction (RESULTS, 3rd section; Fig. 4). Further evidence in support of this conclusion was obtained in a preliminary ESR study which showed that the signal observed for hematin bacterial enzyme at about $g = 5.6$ was reduced by 50–75% during the reaction in the presence of substrate or substrate *plus* ascorbic acid, and by 100% when fully reduced by dithionite. Progressive inhibition by KCN or NaN_3 added during reaction is possibly explicable as due to oxidation of the active heme form of the enzyme by atmospheric O_2 to the inhibitor-sensitive hematin form. The spectral changes observed (in the Soret band) with both enzymes during reaction suggest that the oxygenated heme form of the enzyme may be present during reaction. Clarification of this point must await further studies on the visible spectra with more concentrated enzyme preparations.

Enzyme activity is lost (particularly by the liver enzyme) during storage, but is sometimes recovered during subsequent reaction *i.e.* this deactivation is reversible. Loss of activity during storage is increased by the presence of reducing agents, and minimized by the presence of hematin or substrate (RESULTS, 2nd section). These observations suggest that the sensitive species in this type of deactivation is the apo enzyme and evidence presented for the liver enzyme in Fig. 3 (Curve 2) confirms this. Since the deactivation is reversible, it seems plausible to suggest that it involves conformational changes in the enzyme protein, which are reversible under reaction conditions, *i.e.* in the presence of hematin and tryptophan. The activation effect observed during storage of the Pseudomonas enzyme in the presence of tryptophan may be a related phenomenon. It has been reported elsewhere[14,15] that preincubation with hematin or methemoglobin in the presence of tryptophan increased the tryptophan-pyrrolase activity of liver supernatant. These results, taken together, suggest that both hematin and tryptophan play a role in stabilising the active conformation of tryptophan pyrrolase protein.

ACKNOWLEDGEMENTS

I wish to thank Professor W. EUGENE KNOX for advice and discussion throughout these experiments; Professor ALAN H. MEHLER for providing facilities for molecular weight determinations and for some spectrophotometric measurements; Professor FRANCOIS GROS and Dr. D. H. HAYES for advice and help in the preparation of the manuscript; Dr. HIDEO KON for help in ESR experiments, and Dr. ROBERT BERGER for assistance in measurements with the rapid flow spectrophotometer. It is a pleasure to acknowledge the expert technical assistance of Miss EVELYN STELLA.

REFERENCES

1 T. TANAKA AND W. E. KNOX, *J. Biol. Chem.*, 234 (1950) 1162.
2 O. GREENGARD AND P. FEIGELSON, *J. Biol. Chem.*, 237 (1962) 1903.
3 K. TOKUYAMA AND W. E. KNOX, *Biochim. Biophys. Acta*, 81 (1964) 201.
4 K. TOKUYAMA, W. E. KNOX AND E. STELLA, *Federation Proc.*, 23 (1964) 163.
5 P. FEIGELSON, Y. ISHIMURA AND O. HAYAISHI, *Biochem. Biophys. Res. Commun.*, 14 (1963) 96.
6 P. FEIGELSON, Y. ISHIMURA AND O. HAYAISHI, *Tryptophan Metabolism, Tanabe Amino Acid Res. Found. Symp. Japan, 1963*, Vol. 1, Tanabe Amino Acid Res. Found., Osaka, 1964. p. 19.
7 P. FEIGELSON, *Biochim. Biophys. Acta*, 92 (1964) 187.
8 P. FEIGELSON, *Federation Proc.*, 23 (1964) 163.

9 W. E. KNOX, in S. P. COLOWICK, AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 246.
10 V. H. AUENBACH AND W. E. KNOX, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 621.
11 E. W. SUTHERLAND, C. F. CORI, R. HAYNES AND N. S. OLSEN, *J. Biol. Chem.*, 180 (1949) 825.
12 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 314 (1940) 149.
13 K. KOIKE AND S. OKUI, *Biochim. Biophys. Acta*, 81 (1964) 602.
14 W. E. KNOX, M. PIRAS AND K. TOKUYAMA, *J. Biol. Chem.*, 241 (1966) 297.
15 W. E. KNOX, M. PIRAS AND K. TOKUYAMA, *Enzymol. Biol. Clin.*, 7 (1966) 1.