EFFECT OF AVIDIN ON VERTEBRATE CARBAMYL PHOSPHATE SYNTHETASES

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Avidin does not inhibit the ammonia- and acetylglutamate-dependent carbamyl phosphate synthetase of frog liver (CPSI) or the glutamine utilizing carbamyl phosphate synthetase of pigeon liver (CPSII). It is unlikely therefore that these enzymes require biotin as a cofactor.

Since the synthesis of carbamyl phosphate is a reaction involving the fixation of bicarbonate (1, 2) or carbon dioxide (3), it was logical after discovery by Lynen et al.(4) that biotin functioned as a cofactor in carboxylation reaction to inquire if it was a cofactor for a carbamyl phosphate synthesis. Ravel et al.(5) demonstrated conclusively that biotin was not a cofactor for carbamyl phosphate synthesis by carbamyl phosphokinase. Other laboratories tested the ability of avidin to inhibit the ammonia and acetylglutamate dependent activity of ureotelic vertebrate livers (CPSI) and found that avidin inhibition could not be observed (6, 7, 8, 9); however this negative experimental data has not yet been published.

Wellner et al.(10) have recently published data which indicate that the glutamine-utilizing carbamyl phosphate synthetase (CPSII) of Escherichia coli may be a biotin enzyme, and have suggested that the vertebrate CPSI and II might also be biotin enzymes.

We wish to present here recent experiments (Table I) which show that the unpurified pigeon (or vertebrate) CPSII is not inhibited by avidin, and also the earlier unpublished experiments (6) which demonstrate that 35% pure frog liver CPSI is also not inhibited by avidin (Table II).

Results and Discussion: Table I shows that avidin does not inhibit pigeon liver CPSII. The enzyme is inhibited, as expected, by O-carbamyl-L-serine

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TABLE I

Effect of Avidin on the CPSII Activity of Pigeon Liver Extracts

Additions	CPSII Activity Remaining After Various Pre-incubation Intervals.+		
	10min.	30min.	60min.
1. None	105	94	78
OCS*	46	38	27
2. Avidin	115	101	81
Avidin + OCS*	43	38	34
3. Avidin + Biotin	112	110	85
Avidin + Biotin + OCS*	46	40	33

+ All values are counts/min/μmole citrulline (i.e. carbamyl phosphate) as described earlier (13).

* OCS = O-carbamyl-L-serine, which competively inhibits CPSII with respect to the substrate, glutamine (13).

CPSII activity was analyzed as described earlier utilizing ornithine and purified ornithine transcarbamylase (specific activity of 27,000) from Streptococcus faecalis ATCC #8043 (6) to convert the ^{14}C-carbamyl phosphate formed from ^{14}C-bicarbonate by CPSII to ^{14}C-citrulline. The CPSII preparation is the soluble supernatant of a pigeon liver homogenate after passage over a Sephadex G-25 column to remove the glutamine in the homogenate (13). The activity observed in the absence of added glutamine is 10 counts/min./μmole citrulline.

Twenty mg of avidin (Worthington Biochemicals, containing 11.9 units/mg) was dissolved in 1 ml of M phosphate buffer, pH 7.5. Twenty mg of biotin was dissolved in 1 ml of M phosphate buffer, pH 7.5.

Three vessels containing 800μmoles of phosphate buffer, pH 7.5, in a total volume of 0.8 ml were incubated for 5 minutes at 37°. Vessel 1 contained buffer only; vessel 2 contained buffer and 2 mg of avidin; vessel 3 contained buffer plus 2 mg avidin and 4 mg biotin. At the end of the 5 minutes incubation, 4 ml pigeon liver extract were added to each vessel. Samples (0.6 ml) were taken from vessels 1, 2, and 3 after 10, 30, and 60 minutes at 37° and added to the 0.6 ml solution containing the substrates for standard CPSII assay system (13, 14). Glutamine, 0.1mM, was the substrate used as nitrogen donor. A vessel containing 0.1mM glutamine plus 5mM of OCS, a good CPSII inhibitor, was also tested. At the end of the enzyme activity assay the reaction was terminated by the addition of perchloric acid and carrier ^{12}C-citrulline. The citrulline was purified analyzed and counted as described earlier (13).

(OCS) and avidin addition does not affect the inhibitory action of this glutamine analog. The amounts of avidin (24 units) used in the experiment of

Table I are estimated to be 240-fold the amount of avidin required to complex with the 0.1µg of biotin expected in 4.0 ml of the 100,000xg supernatant of a 1 to 10 homogenate of pigeon liver. This assumes that pigeon liver like chicken (9) and turkey (11) liver contain 1µg of biotin/g. wet weight of liver, and that 25% of the total biotin in the pigeon liver homogenate is associated with the soluble supernatant protein as it is in chicken liver (9).

TABLE II

Effect of Avidin on a Frog Liver Extract Containing CPSI and a Biotin-Dependent Enzyme Catalyzing an ATP - ^{32}Pi Exchange Reaction.

Preincubation Addition	CPSI Activity	ATP - ^{32}Pi Exchange Activity
	µmole carbamyl phosphate	cpm/µmole ATP
1. None	0.87	245
2. Avidin	0.79	40
3. Avidin + Biotin	0.81	225
4. No frog extract	0.00	23

The frog enzyme used was extracted from sucrose mitochondria as described earlier (2). This extract is 35% pure CPSI (6). The CPSI assay used is the phosphate assay described previously (6). The enzyme (20µg) was preincubated for 10 minutes at 37° in 0.2 ml solution containing the 50µmoles Tris buffer, pH 8.0, used for the enzyme assay, either with 50µmoles of KCl, or 0.1 mg avidin (Nutritional Biochemical Co.) plus 50µmoles KCl, or with 0.1 mg avidin and 30µmoles of biotin plus 50µmoles of KCl before the substrates were added. At the end of the preincubation, the substrates for CPSI were added in quantities previously described (6).

For the ATP-^{32}Pi exchange reaction, the same frog extract (200µg protein) was incubated at 37° for 10 minutes in 0.55 ml containing 50µmoles Tris buffer, pH 8.0, either with 100µmoles of KCl; or 0.8 mg avidin (Nutritional Biochemical Co.) plus 100µmoles of KCl; or 0.8 mg avidin and 300µmoles biotin plus 100µmoles of KCl. After the preincubation, 0.2 ml of solution containing 6µmoles of ATP, 8µmoles of glutathione, 6µmoles of magnesium chloride and 6µmoles of potassium ^{32}Pi, pH 7.5, containing 1.7 x 10^6 counts/min. was added and the incubation was continued for 30 minutes at 37°. The reaction was terminated by the addition of two volumes of 5% trichloroacetic acid. The ATP was absorbed on charcoal which was washed with ^{31}P-orthophosphate solution followed by several distilled water washes to remove ^{32}Pi substrate. The charcoal-absorbed ATP was hydrolyzed with N HCl for 15 minutes at 100° and aliquots containing the ^{32}Pi, so released, were plated on planchetts and counted with a Geiger-Mueller counter.

Table II is the earlier unpublished experiment showing that avidin does not inhibit CPSI of the frog liver (6). The preparation is a $5x10^{-3}$M glutathione extract of the washed 3000xg particulate of a frog liver homogenate (2). The enzyme in this simple extract is 33-37% pure. It does, however, contain an unidentified biotin-enzyme which catalyzes the ^{32}Pi-ATP exchange reaction in the presence of bicarbonate ion, a reaction typical of biotin enzymes (12). We were fortunate that the biotin enzyme was present in this extract because it establishes that although the avidin added was sufficient to inhibit a biotin enzyme completely, the same avidin had only a small inhibitory effect on CPSI. Secondly, the preincubation of biotin with avidin restores the ^{32}Pi-ATP exchange reaction, but does not significantly reverse the small inhibitory effect of avidin on CPSI. The ratio of frog liver protein/avidin/biotin was maintained nearly constant in the two experiments although the absolute amounts of all three components were changed since the ^{32}Pi-ATP exchange activity is very weak while CPSI activity is very potent and therefore requires much less protein.

The avidin preparation used in Table II also did not inhibit the CPSII activity of pigeon liver extracts.

These results are not consonant with either frog liver CPSI or, more particularly, with pigeon liver CPSII, the vertebrate enzyme most closely related to the *E. coli* enzyme studied by Wellner *et al.* (10), being biotin enzymes. Although both experiments suffer from the criticism that they are negative, we are unaware of any biotin enzyme that is not sensitive to avidin inhibition. However, the opposite corollary, i.e. that an enzyme inhibited by avidin is a biotin enzyme, is not always correct, for avidin is a basic protein which can complex with and inhibit anionic proteins.

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