SEPARATION AND DIFFERENTIAL SENSITIVITY TOWARD AVIDIN OF CARBAMYL PHOSPHATE SYNTHETASE AND UREA AMIDOLYASE

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1. Introduction

In a recent communication from this laboratory [1], we have described a new, inducible enzyme, ATP:urea amidolyase (UALase), from urease-negative yeast and uni-cellular green algae, which catalyzes the reaction

$$\text{urea} + \text{ATP} \xrightarrow{Mg^{++}, K^{+}} CO_2 + 2NH_3 + \text{ADP} + P_i .$$

This enzyme is remarkably sensitive to inhibition by highly purified egg white avidin, a phenomenon which can be completely prevented by inclusion of excess biotin in the assay system. Inasmuch as avidin has been found to selectively inhibit all known biotin-enzymes and, indeed, is now accepted as a diagnostic tool for the detection of such reactions [2], the proposal was made that UALase belongs to that class of enzymes which contain biotin in the form of a bound, functionally-active prosthetic group.

A recent publication by Wellner, Santos and Meister [3], asserting that the glutamine-dependent carbamyl phosphate synthetase (CPSase, EC 2.7.2.5) of *Escherichia coli* may also be a biotin-enzyme, has focused our attention on the relevant question of whether these two enzymic processes might possibly represent analogous activities of the same protein molecule. At this time we wish to report results which indicate that not only is each reaction catalyzed by a different, separable protein entity, but also that the glutamine-dependent CPSases present in extracts of three widely-different organisms – a yeast (*Candida utilis*), a bacterium (*Aerobacter aerogenes*), and a fungus (*Agaricus bisporus*) – do not appear to be biotin-enzymes.

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2. Materials and methods

2.1. *Source of organisms and preparation of enzymes*

Candida utilis (ATCC No. 8205) was harvested after growth on urea as sole nitrogen source and disrupted by sonic oscillation as described [4]. The crude extract was carried through step 3 (ammonium sulfate fractionation) of the scheme for purification of UALase [4], and the resulting preparation was employed directly for gel-filtration as described in fig. 1.

Agaricus bisporus (the mushroom of commerce in the U.S.) was obtained from a local food store. In the manner described previously [5], an extract was prepared from 100 g of sporophores and concentrated in CPSase activity by precipitation of proteins with ammonium sulfate. The precipitate was washed with cold, 75% saturated ammonium sulfate and dissolved in 6 ml of 0.02 M potassium phosphate buffer, pH 7.5. After clarifying the material by centrifugation, the supernatant was used directly in the experiment described in fig. 2.

A strain of *Aerobacter aerogenes* (obtained from the culture collection of the Department of Microbiology, The University of Michigan) was grown aerobically on Enterobacter medium No. 48 [6]. Cells were

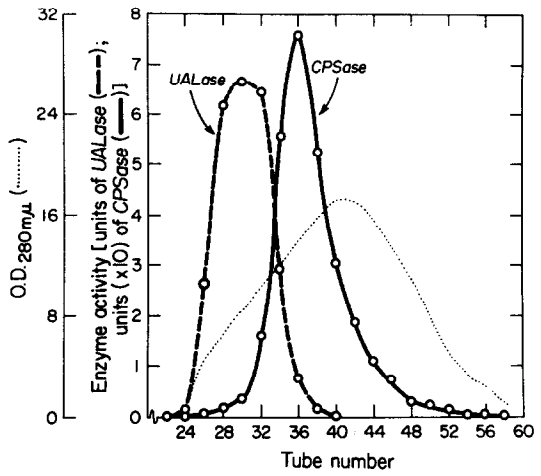

Fig. 1. Separation of *C.utilis* UALase and CPSase by gel-filtration on Sephadex. The yeast protein fraction (see section 2.1) was dissolved in 12 ml of a chilled solution of 0.02 M potassium phosphate buffer, pH 7.5, containing 10% (v/v) glycerol (Buffer A), and placed on a column (3 × 50 cm) of Sephadex G-200 previously equilibrated against this same solution. Elution with Buffer A was carried out at 3°, 5 ml fractions being collected at 20 min intervals. Enzyme activities (units expressed as micromoles of product formed per min per fraction) were determined as given in section 2.2, using aliquots of suitable size taken from alternate tubes.

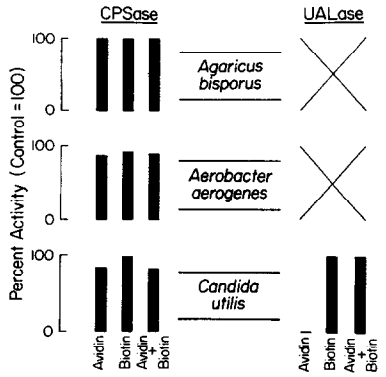

Fig. 2. Differential sensitivity toward avidin of UALase and glutamine-dependent CPSases from yeast, fungal and bacterial sources. The standard incubation systems referred to in section 2.2 were employed, with supplements of 200 μg of avidin and/or 100μg of biotin included as indicated. A mixture of the contents of tubes 28 and 38 from the Sephadex column eluate (see fig. 1) was used as the source of the *C. utilis* enzymes; the remaining preparations were obtained as given in section 2.1. Control values (i.e. enzyme activities in the absence of avidin and biotin) were as follows (in micromoles of carbamyl phosphate or CO_2 formed per hr per mg protein): 27.6 for UALase from *C. utilis*; 0.35, 0.12, and 0.08 for CPSase from *C. utilis, A. aerogenes,* and *A. bisporus,* respectively. Extracts of the latter two organisms contained no detectable UALase activity.

centrifuged in late log phase, suspended in 0.05 M potassium phosphate buffer, pH 7.5, and disrupted by sonic oscillation. The extract was centrifuged at 20000 × *g* and the supernatant was treated with solid ammonium sulfate to 67% saturation. The resulting precipitate was dissolved in 0.1 M tris-HCl buffer, pH 7.5, containing 10 mM KCl, 10 mM $MgSO_4$, and 5 mM β-mercaptoethanol, dialyzed overnight versus two changes of this same solution, and assayed directly for glutamine-dependent CPSase activity.

Ornithine transcarbamylase (OTCase, EC 2.1.3.3) was prepared as described in ref. [5].

2.2. *Enzymatic assays*

UALase activity was determined by the radioactive technique previously described [1], which is based on the rate of release of $^{14}CO_2$ from ^{14}C-urea under a standard set of conditions. In the avidin inhibition studies, glutamine-dependent CPSase activity was measured by the isotopic procedure [5]. For detecting this enzyme in effluents from the Sephadex column, the colorimetric method for citrulline determination was employed [7]. In either case incubations were carried out in the presence of an excess of ornithine and OTCase.

2.3. *Chemicals*

^{14}C-urea was purchased from Schwarz BioResearch, ^{14}C-sodium bicarbonate from New England Nuclear Corp., *d*-biotin from Hoffman-LaRoche, highly purified avidin (12 units per mg) from Worthington Biochemical Corp., and Sephadex from Pharmacia Fine Chemicals, Inc.

3. Results

In fig. 1 is shown the separation of UALase from CPSase which can be achieved by passage of the *C. utilis* enzyme preparation over a column of Sephadex

G-200. Although previous studies [1, 8] had indicated that highly purified samples of yeast UALase and *E. coli* CPSase were not contaminated by the catalytic activities of one another, the results shown here clearly establish that, within the same cell, one is dealing with distinctly separable proteins.

The differential effect of avidin on the two enzyme activities in *C. utilis* is illustrated in fig. 2, along with data from similar studies on the glutamine-dependent CPSase activities of *Aerobacter aerogenes* * and *Agaricus bisporus*. The comparatively high level of avidin employed resulted in total inhibition of UALase activity; prior inclusion of excess biotin, although without effect on the uninhibited reaction, could completely nullify the inhibitory property of avidin. In contrast, CPSase remained either unaffected by this same high level of avidin (*A. bisporus*) or suffered only a 16% loss in activity (*A. aerogenes, C. utilis*). *This small inhibition could not be prevented by the presence of biotin.*

4. Discussion and conclusions

From a structural standpoint, the urea molecule can be looked upon as a nitrogen-system counterpart of carbonic acid, wherein –OH groups are replaced by $-NH_2$. Hence it may not be unreasonable to speculate that an analogy might exist between the mechanisms of urea cleavage and of carbon dioxide activation catalyzed, respectively, by UALase and all of the five known biotin-contining carboxylases [2]. Providing that biotin were involved in the CO_2-fixing activity of CPSase, such an analogy could be extended to include this enzyme as well. Indeed, one might envision even a closer connection between CPSase and UALase inasmuch as each of these enzymes, in contrast to the carboxylases, concerns a reaction dealing primarily with the metabolism of the carbon-nitrogen bond and, furthermore, one in which the presence only of cosubstrate (i.e. bicarbonate or urea) is required to effect the cleavage of ATP [1, 10]. The sequence of intermediate steps proposed for the action of CPSase includes the formation of an enzyme-bound carbamyl (or carbamate) moiety [11]. If this were linked to the functional N-(1) position of the biotin residue, as is CO_2 in the carboxylases [10], the resulting bound intermediate would essentially be identical to that favored by us, purely on theoretical grounds, for the UALase-catalyzed reaction. For these reasons it was considered crucial to ascertain (a) whether UALase and CPSase might conceivably be one and the same protein, and (b) whether biotin is indeed a functionally-active component of *C. utilis* CPSase.

Our results indicate that yeast UALase and CPSase are activities associated with distinctly different proteins, as shown by the facile separation of these enzymes on a column of Sephadex G-200. Examination of the effects of avidin on each activity under essentially identical conditions has revealed further fundamental differences. No evidence could be obtained from avidin inhibition studies to implicate biotin in the catalytic activity of glutamine-dependent CPSase originating from either a yeast, bacterial, or fungal source. In contrast, yeast UALase, as reported previously [1], could be inhibited completely by avidin under these same conditions.

A number of other reports have recently appeared which likewise are at variance with the conclusions drawn by Wellner et al. [3]. Peng and Jones [12] could find no significant effect of avidin on either frog liver (ammonia-dependent) or pigeon liver (glutamine-dependent) CPSase systems. Huston [13] has reported similar results with beef liver (ammonia-dependent) CPSase and, furthermore, could obtain no evidence that biotin was present in the purified *E. coli* enzyme or that avidin inhibited its activity. Guthöhrlein and Knappe [14] have noted a similar result with purified rat liver (ammonia-dependent) CPSase. The present observations are in line with these reports. They have forced us to reconsider certain thoughts we may have entertained as to the possible similarity in mechanism between the reactions catalyzed by UALase and CPSase. Studies currently in progress, using larger quantities of more highly purified preparations of UALase, hopefully will aid in clarifying the functional role of biotin in this enzyme.

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* Urease (EC 3.5.1.5) is present in extracts of this organism when cells are grown on urea as sole nitrogen source [9].

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