Haemocyanin Synthesis in the Crayfish, *Astacus leptodactylus: in vitro* Production by the Midgut Gland after Addition of Oxygen Binding Modulators and Exogenous Copper

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Haemocyanin synthesis in the midgut gland of the crayfish, *Astacus leptodactylus,* was studied *in vitro*. The addition of exogenous copper into the culture medium reduces the synthesis of total proteins and of haemocyanin. L-lactate, uric acid and dopamine, which modify the affinity of haemocyanin towards oxygen, do not influence the biosynthesis of the polypeptide.

Introduction

The copper containing oxygen carrier haemocyanin is the predominant protein in the hemolymph of most molluscs, crustaceans and spiders. Despite its abundance and the detailed knowledge on physiological aspects, relatively little information exists on haemocyanin synthesis and its regulation [1–4]. In analogy to haemoglobin, where the addition of haeme influences the haemoglobin production [5], copper has been discussed as a limiting factor for haemocyanin synthesis, although its role in haemocyanin biosynthesis is unclear till now [2]. In a previous study we found a decrease in haemocyanin production by the midgut gland of the crayfish *A. leptodactylus* after three days of incubation in a medium without copper [6]. The question arose whether copper might be a rate limiting factor for haemocyanin synthesis. Knowledge of the concentrations of copper in the midgut gland and its secretion into the medium during the *in vitro* incubation as well as an investigation of the influence of exogenous copper on haemocyanin synthesis and secretion should shed some light on the role of copper in haemocyanin biosynthesis.

In recent years it has been shown that L-lactate, uric acid and dopamine can modulate oxygen binding of haemocyanin [7–9], but it is not known so far whether these modulators also exert an influence on haemocyanin synthesis. We therefore investigated this positive influence of the modulators on the production of haemocyanin in the midgut gland of the crayfish, *Astacus leptodactylus,* which has been identified as the only site of haemocyanin synthesis in this species [10].

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Materials and Methods

In the present study we used an established organ culture system for crayfish tissues [6] and techniques for the quantitative measurements of haemocyanin synthesis and secretion described elsewhere in detail [11]. In short, incorporation of [^{35}S]methionine into total proteins (precipitation by trichloro-acetic acid) and haemocyanin (immuno-precipitation) was measured, as well as the secretion of newly synthesized total proteins and haemocyanin into the medium after 5 h. Copper was determined by atomic absorption spectrometry.

Results and Discussion

To examine our hypothesis that copper is a limiting factor of haemocyanin synthesis we determined its concentration in haemolymph, midgut gland and in the culture medium after 5 and 24 h of incubation. We found 40 µg of copper per ml of haemolymph and a 4-fold higher concentration in the midgut gland. After 5 h of incubation only 2.3%, after 24 h 4.3% of the total copper content of the midgut gland is secreted into the medium (Table I). A concentration of 40 µg haemocyanin per ml of haemolymph is comparable to the concentrations which have been determined for *Astacus leptodactylus* and two nearly related species be-

Table I. Copper concentrations in haemolymph and the midgut gland of male intermoult *Astacus leptodactylus*. Copper was determined by atomic absorption spectrometry. The values are means and standard deviations of 4 determinations.

Organ	Copper concentration (m ± S.D.)
Haemolymph	40.1 ± 7.4 µg/ml
Midgut gland	152.5 ± 44.4 µg/g fr. wt.
Medium	
after 5 h of incubation	3.5 ± 0.1 µg/g fr. wt.
after 24 h of incubation	6.5 ± 0.2 µg/g fr. wt.

fore [12, 13]. The 4-fold higher copper concentration in the midgut gland in comparison to the haemolymph indicates a possible function as storage organ for copper. Similar high concentrations have been found in various other crustacean species [14–19]. Since less than 5% of total copper content is secreted into the medium after 24 h of incubation, copper does not seem to be a rate limiting factor for haemocyanin synthesis under our experimental conditions. A second set of experiments is also in favour of this hypothesis: The addition of exogenous copper does not increase haemocyanin synthesis as one might suspect, if copper would be a limiting factor. On the contrary, the supplementation of the medium with copper reduces haemocyanin and total protein synthesis (Fig. 1). It is also evident from these results, that the relative decrease of haemocyanin in prolonged incubations occurs independent of copper addition. This might be the reason for our earlier observations that only traces of newly synthesized haemocyanin were detected after 3 days of incubation [6].

In a second set of experiment, the midgut gland was incubated in a medium supplemented with either uric acid (1 mM), L-lactat (10 mM) and dopamine (0.1, 1 and 10 µM; data not shown). These concentrations are effective in modulating oxygen binding of haemocyanin. Addition of these compounds did not result in a significant difference in the rate of synthesis (Fig. 2a) and secretion (Fig.

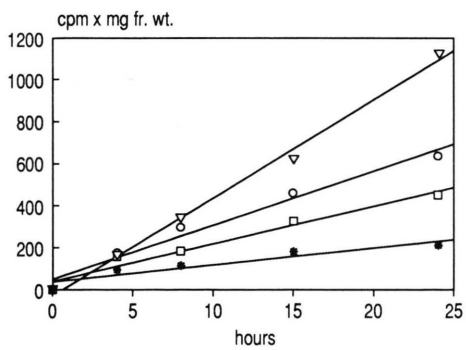

Fig. 1. *In vitro* secretion of newly synthesized total proteins (▽ control, ○ experimental) and haemocyanin (□ control, ★ experimental) from the midgut gland of the crayfish, *Astacus leptodactylus*. The midgut gland of an animal was divided into a control and an experimental (containing 1 mM copper sulphate) part and incubated in a medium containing [^{35}S]methionine. At the times given aliquots of the medium were taken and the secretion of newly synthesized total proteins was determined after precipitation with 5% trichloroacetic acid (final concentration) and haemocyanin was quantified after immunoprecipitation with a polyclonal anti-haemocyanin antibody.

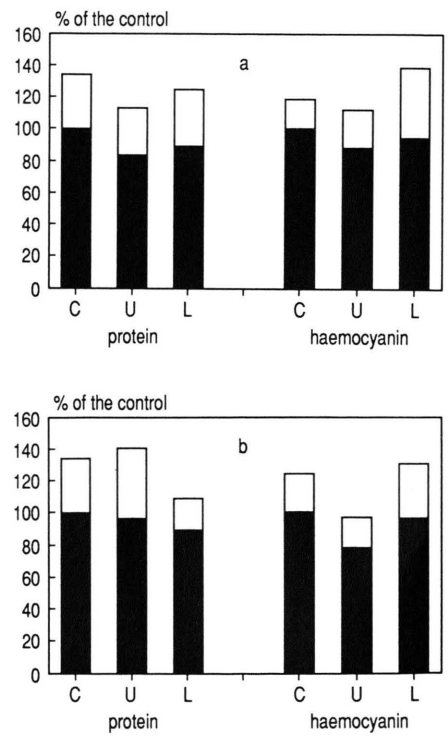

Fig. 2. Influence of the oxygen binding modulators uric acid (1 mM) and L-lactate (10 mM) on haemocyanin synthesis (Fig. 2a) and secretion (Fig. 2b) in the midgut gland of the crayfish, *Astacus leptodactylus*. The midgut glands of the animals were divided into two halves and incubated for 5 h in [^{35}S]methionine containing medium in the presence or absence of the corresponding compound. The values represent means and standard deviations (⊓) of three independent experiments. C, control; U, uric acid and L, L-lactate.

2b) of both total proteins and haemocyanin. Thus, these compounds act only as allosteric modulators of haemocyanin oxygen binding but do not influence its synthesis.

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