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Histidase from the unicellular green alga *Dunaliella tertiolecta*: purification and partial characterization

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In summer, the concentrations of dissolved organic nitrogen compounds are often higher than that of inorganic nitrogen. Under such conditions it would be advantageous if phytoplankton species could utilize organic nitrogen sources, including free or combined amino acids, in addition to inorganic nitrogen. This study focused on histidine, the degradation of which potentially yields three nitrogen atoms for each molecule of histidine. In this work, histidase from *Dunaliella tertiolecta*, a deaminating enzyme catalysing the first steps of histidine degradation, was purified 4000-fold and partially characterized. The molecular weight of the native enzyme was estimated to be 155 kDa, corresponding to four subunits of 38 kDa. *D. tertiolecta* histidase is stable in the presence of dithiothreitol and is inactivated by cyanide. Histidinol phosphate, histidine and Mn^{2+} are effective protectors against cyanide inactivation. The enzyme did not exhibit classical Michaelis-Menten kinetics but showed a relationship between the rate of catalysis (V) and the concentration of substrate (S) that was characteristic of negative allosteric behaviour. A Hill coefficient of 4 was measured for histidine concentrations higher than 22.5 mM. Guanine, xanthine and cytosine nucleotides are inhibitors of *D. tertiolecta* histidase.

Key words: characterization, *Dunaliella tertiolecta*, histidase, microalgae, purification

Introduction

One of the important macronutrients controlling phytoplanktonic growth is nitrogen which, in seawater, is available mainly as inorganic nitrogen (MacCarthy, 1980; Glibert, 1988). During the spring, most of the nitrogen in seawater is present as nitrate. Autotrophic and heterotrophic organisms using nitrate for growth convert it into ammonium and then into the different forms of organic nitrogen. In summer, as a consequence of the phytoplankton bloom, nitrate is depleted in large parts of the surface waters (MacCarthy, 1980; Glibert, 1988) and the concentrations of dissolved organic nitrogen compounds are then often higher than that of inorganic nitrogen (Braven *et al.*, 1984; Antia *et al.*, 1991). Under such conditions it would be advantageous for phytoplanktonic species to have the ability to use organic nitrogen also, a large portion of which consists of dissolved free amino acids and dissolved combined amino acids (Antia *et al.*, 1975; Flynn & Butler, 1986).

Many species of algae have been found to grow in laboratory culture on amino acids and other organic nitrogen sources. Direct evidence for net influx of 14–18 different amino acids (at nanomolar concentrations) into phytoplankton cells was obtained by HPLC techniques for *Phaeodactylum tricornutum* and *Tetraselmis subcordiformis* (Qafaiti & Stephens, 1984, 1989). Intracellular accumulation of single, exogenously supplied amino acids has frequently been described, such as that of several amino acids by *T. subcordiformis* (North & Stephens, 1969) and L-lysine by *P. tricornutum* (Flynn & Syrett, 1986), while mixtures of exogenous amino acids have been reported to support the synthesis of proteins and other complex polymers required for the heterotrophic growth of Antarctic diatoms under light-limiting conditions (Rivkin & Prett, 1987). A novel mode of amino acid utilization by some prymnesiophytes and dinoflagellates involves a cell surface deaminase that converts L-amino acid to NH_4^+, H_2O and the corresponding α-keto acid, with subsequent uptake and assimilation of the released NH_4^+ (Palenik & Morel, 1990; Palenik & Morel 1991; Pantoja & Lee, 1994). However, for most algae that lack the extracellular deaminase, the absorbed amino acids are generally assimilated and often utilized as nitrogen sources for growth with the peculiarity that amino acids can serve as both nitrogen and carbon sources and are therefore under a complex control network. In many instances, however, the enzyme systems involved in the uptake, degradation and assimilation of specific amino acids have not been fully separated, purified or characterized.

Histidine catabolism has been studied in microorganisms such as *Bacillus sphaericus* (Ahmed *et al.*, 1993), *Bacillus subtilis* (Atkinson *et al.*, 1990), *Pseudomonas putida* (Allison & Phillips, 1990) and *Pseudomonas testosteroni* (Coote & Hassal, 1973). In these organisms histidase, a deaminating enzyme catalysing the first step of histidine degradation, has been purified and characterized. No data are available regarding histidine catabolism in algae.

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In marine natural waters, the average concentration of histidine recorded is around 0.08 mM (Kirchman & Hobson, 1986), but histidine can occur in patches where concentrations of histidine and other amino acids can be several orders of magnitude greater (Braven *et al*., 1984). In coastal waters and in estuaries, high concentrations of free and bound organic substances are also found. Under such conditions it would be advantageous for many phytoplankton species to have the ability to use organic nitrogen sources in addition to inorganic nitrogen. For *Dunaliella tertiolecta* we have previously shown (Hellio & Le Gal, 1998) that when ammonium has been depleted from the medium, this unicellular alga is able to take up histidine and to grow on it as the sole source of nitrogen. In such conditions *D. tertiolecta* expresses histidase (EC 4.3.1.3;8), the enzyme that converts L-histidine to urocanate and ammonium. This paper reports the purification of histidase from *D. tertiolecta* and its partial characterization.

Materials and methods

Organism and culture

Dunaliella tertiolecta Butcher (CCAP 19/6B) was obtained directly from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, PA34 4AD, Scotland. The cells were grown axenically in 15 l batch cultures at 18 °C under continuous illumination of 150 μmol m^{-2} s^{-1} from white fluorescent lamps. The sterile growth medium consisted of natural seawater taken from the open Atlantic Ocean (salinity = 32.5 PSU), enriched with f/2 medium of Guillard & Ryther (1962) but without the nitrogen source, thiamin, biotin and vitamin B_{12}. Nitrogen sources consisting of both NH_4Cl and histidine were added separately. The medium was sterilized by autoclaving at 120 °C and 1 bar.

Cell growth was followed by direct cell counting using a Malassez haemocytometer. Ammonium concentration was measured according to Solarzano (1969) and histidine was estimated colorimetrically according to Tabor (1957).

Harvesting of the cells and preparation of the crude extract

Batches of 15 l of culture were grown to the stationary phase, where histidase activity was found to be maximal. The culture was carried out in two steps as previously described (Hellio & Le Gal, 1998). During the first phase of growth, in 7.45 mM NH_4Cl for 4 days, cell biomass reaches 8×10^5 cells l^{-1}. The second phase of growth, which utilizes histidine (0.865 mM), begins only when ammonium is approaching exhaustion (< 4 mM). This procedure ensures the production of a sufficient amount of histidase-induced cells. Prior to harvesting, cells were pretreated with a cryoprotective agent according to the method of Maurin & Le Gal (1997): for 12 h before harvesting, the cells were exposed to 3 g l^{-1} sucrose that was aseptically added to the growth medium. The cells were harvested by centrifugation (3300 *g*, 4 °C, 20 min) and the resulting pellet was equilibrated for 20 min in 15 ml of extraction buffer (buffer A: Tris 0.02 M, pH 7.8, 1 mM benzamidine, 5 mM e-aminocaproic acid, 0.5 mM dithiothreitol, 200 mM phenylmethylsulphonylfluoride (PMSF), 2.5 mM Mn^{2+}). The concentrated cell suspension was then centrifuged (5000 *g*, 4 °C, 20 min) and the concentrated pellet was used immediately or preserved by freezing at −20 °C. Freezing does not affect enzyme activity.

The cell pellet (1 g wet weight) was disrupted with the help of an Ultraturax homogenizer for 4×30 s, at full speed, in 3 ml of buffer A at 4 °C. PMSF was added directly to the homogenate at a final concentration of 0.5 mM. The homogenate was clarified by two successive centrifugations at 17000 *g* and 4 °C for 30 min. The supernatant was used as the crude extract.

Purification of histidase

Histidase was purified from a 15 l batch culture of *Dunaliella tertiolecta* grown on 0.865 mM histidine and 7.45 mM NH_4^+. Cells were harvested after 8 days of growth. The crude extract was prepared as described previously. All subsequent treatments were carried out at 4 °C. Fractionation was achieved by precipitation with ammonium sulphate; solid ammonium sulphate was added to give 70% saturation. After 15 min the precipitate was removed by centrifugation (17000 *g*, 4 °C, 15 min). The supernatant was then treated with ammonium sulphate to 80% saturation and after 15 min the precipitate was collected by centrifugation. The pellet, which contained the enzyme, was resuspended in 10 ml of phosphate buffer (buffer B: 0.02 M, pH 7.6) before being deposited on a column (57 cm × 1.8 cm) of Sephacryl S-300 HR previously equilibrated with buffer B and eluted with the same buffer at a rate of 13 ml per hour. Fractions of 3 ml were collected and those containing enzyme activity were combined. Microgranular DEAE-cellulose was equilibrated with buffer B and packed in a column of 40 cm × 2 cm. The enzyme was first concentrated to 6 ml after filtration on a 1.2 mm filter (Sterile Acrodisc, Gelman Sciences) and concentration by ultrafiltration (Amicon cell, YM 30 membrane), then deposited on the column and washed with 400–600 ml of buffer B. Histidase was eluted with a linear gradient of 100 ml of buffer B and 100 ml of buffer B containing 0.7 M NaCl. The elution rate was 45 ml per hour and 3 ml fractions were collected. The enzyme activity was eluted at 0.4 M NaCl.

The fractions containing histidase activity were pooled and concentrated to a volume of 8 ml and dialysed overnight against phosphate buffer (buffer C: 50 mM, 0.5 M NaCl, pH 7.8). The enzyme extract was then subjected to immobilized metal ion affinity chromatography (IMAC) using an IDA-Sepharose 6 B column (Hajjou & Le Gal, 1994). The column was charged with Cu^{2+}, equilibrated and rinsed with buffer C. Elution of the enzyme was achieved at 4 °C with a glycine gradient (0–1

M) in the same buffer and fractions of 2 ml were collected at a flow rate of 17 ml h^{-1}. The enzyme was eluted with 20 mM glycine.

Molecular mass estimation

The relative molecular mass of histidase was estimated by gel filtration with a Sephacryl 300 HR column (57 × 1.8 cm) equilibrated with buffer B (phosphate buffer 0.02 M, pH 7.6, 0.02 M $MnCl_2$). Blue dextran was used to measure the void volume. Thyroglobulin, apoferritin, alcohol dehydrogenase and bovine serum albumin were used as molecular weight standards to calibrate the column. All molecular weight markers were eluted at a flow rate of 13 ml h^{-1} and collected in fractions of 3 ml.

Electrophoresis

Subunit molecular mass was determined by SDS-PAGE according to the method of Weber & Osborn (1969) using 8 × 10 cm slab gels containing 12% acrylamide. Homogeneity of the enzyme was tested electrogenically using the method of Davis (1964). Protein bands on the gels were silver stained using the method of Morissez (1980).

Assay of enzyme activity

Histidine ammonia lyase activity was measured by following the change in absorbance at 277 nm corresponding to the production of urocanic acid (Tabor & Mehler, 1959). The extinction coefficient of transurocanate at this wavelength is 1.88×10^{-4} M^{-1} cm^{-1}. The standard reaction mixture contained 0.01 M each of L-histidine, HCl and sodium pyrophosphate buffer at pH 9.5, and 10–100 ml enzyme in a final volume of 1 ml. The increase in absorbance was followed at 20 °C. A unit (U) of enzyme activity was computed as micromoles of final reaction product per minute. Specific activity were expressed as the quantity of enzymatic units per milligram of protein (U mg^{-1}).

Protein determination

Total protein was determined in the enzymatic solutions according to the method of Bradford (1976), using Coomassie Brilliant Blue G 250 pure (Serva) and with bovine serum albumin as standard.

Results

Enzyme purification

The result of a typical purification procedure is presented in Table 1. A yield of approximately 50% was obtained with a purification factor of 400 following IMAC chromatography, although it was only about 7.7% after DEAE chromatography. The molecular mass of native histidase was estimated to be 155 kDa by gel filtration on Sephacryl HR-300 (Fig. 1*a*). SDS gel electrophoresis of histidase from *D. tertiolecta* under denaturing conditions gave a molecular mass close to 38.2 kDa (Fig. 1*b*), consistent with the histidase comprising four subunits of 38.2 kDa each.

Stability

The thiol compounds mercaptoethanol, dithiothreitol and cysteine were tested on crude extracts of the enzyme. The results (Fig. 2) indicate that the activity of histidase decreased rapidly with time (56% of the initial activity after 1 day, 11% of the initial level of activity after 2 days) in the presence of mercaptoethanol or cysteine. In the presence of dithiothreitol, histidase activity remained stable for several days.

Effect of cation

A series of cations was tested, following a prior dialysis against 50 mM EDTA (Table 2). No inhibitory effect of EDTA was recorded. Mn^{2+} is a slight activator of histidase activity. Co^{3+}, Fe^{3+}, Fe^{2+} and Cu^{2+} are inhibitors.

Kinetic properties

The substrate saturation curves of histidase did not exhibit classical Michaelis-Menten profiles (Fig. 3). In contrast, a biphasic curve of the rate of catalysis (V) versus the concentration of substrate (S) was observed. Plotting the corresponding data according to Hanes-Wolf (Fig. 4*a*) yielded two values for the maximal rate (V_m) and two values for the Michaelis constant (K_m) respectively: one pair of values was obtained for substrate (histidine) concentrations up to 22.5 mM, and a second pair was obtained when histidine concentrations were higher than 22.5 mM. Accordingly, the representation of Hill (Fig. 4*b*) leads to two different values for the Hill coefficient (n) depending on whether the histidine concentration is higher or lower than 22.5 mM.

When the same experiments were performed following a heating step (35 °C, 10 min), the substrate saturation curve (Fig. 5) exhibited a classical Michaelis-Menten profile. Plotting the corresponding data according to Hanes-Wolf (Fig. 5*b*) yielded only one value for V_m and K_m. The results are summarized in Table 3.

Effectors

The activity of purified histidase was tested in the presence of different potential effectors (Table 4). NH_4^+ at different concentrations exerted no effect. Histidase was inhibited by cyanide (Fig. 6). The results show that histidinol phosphate 50 mM, L-histidine 5 mM and Mn^{2+} 5 mM offered protection against inactivation by cyanide.

Effect of nucleotides

Several nucleotides were found to exert a strong inhibitory effect on histidase (Table 5). Guanine, xanthine and

Table 1. Summary of the purification of *D. tertiolecta* histidase.

Step	Total activity (U) × 10^{-4}	Specific activity (U mg^{-1}) × 10^{3}	Yield (%)	Purification factor
Crude extract	50.97	7.22	100	1
Pellet after precipitation with 80% ammonium sulphate	12.18	11.60	24	2
Gel filtration	10.68	41.10	21	6
DEAE cellulose	4.78	384.52	8	55
IMAC	25.99	3050.05	51	434

Histidase was purified from a 15 l batch culture of *Dunaliella tertiolecta* grown on 0.865 mM histidine and 7.45 mM NH_4^+. Cells were harvested after 8 days of growth.
IMAC, metal ion affinity chromatography.

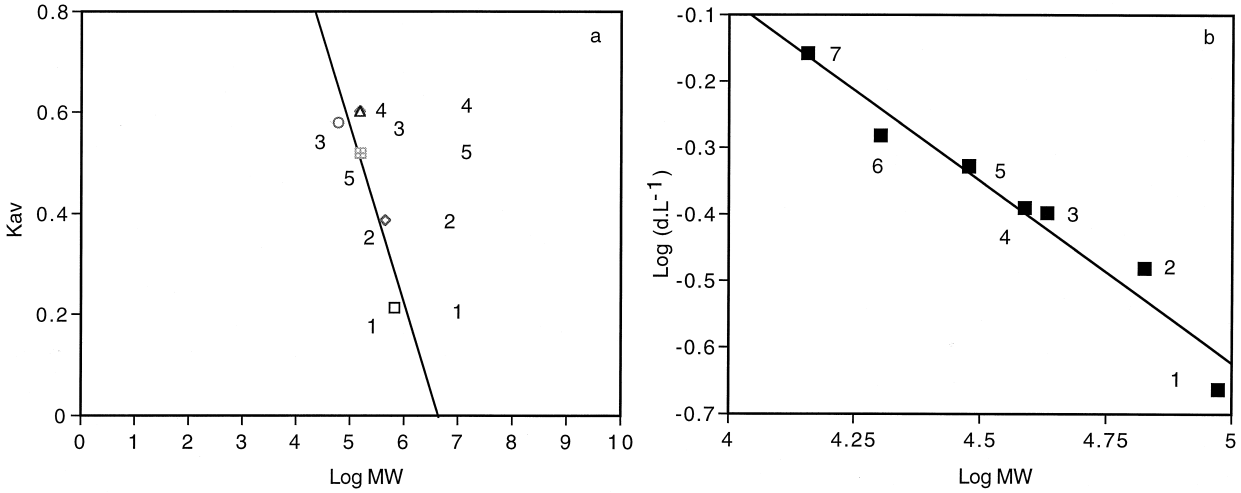

Fig. 1. Calibration curve from the Sephacryl column. (*a*) Molecular mass determination of *D. tertiolecta* histidase. The molecular markers are (1) thyroglobulin, (2) apoferritin, (4) bovine serum albumin and (5) alcohol dehydrogenase. (3) is the native histidase. (*b*) SDS gel electrophoresis. Determination of the mass of the histidase subunit. Electrophoresis was performed under denaturating conditions. The molecular markers are respectively (1) phosphorylase b, (2) bovine serum albumin, (3) ovalbumin, (4) carbonic anhydrase, (6) soybean trypsin inhibitor, and (7) alpha lactoalbumin. (5) is the histidase subunit.

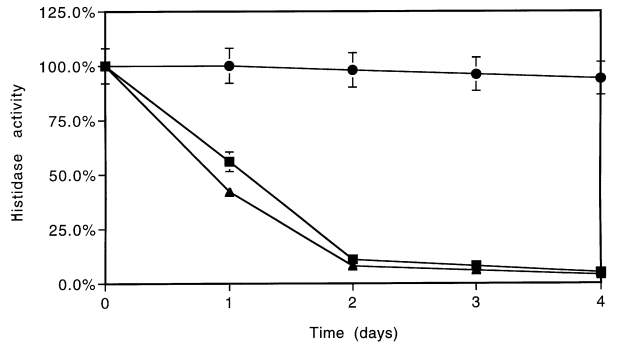

Fig. 2. Effect of thiol compounds on histidase activity conservation. Crude extract was prepared by sonication of 1 g of algae in 3 ml of buffer without thiol compounds; 15 mM mercaptoethanol (squares), 5 mM dithiothreitol (circles) or 5 mM cysteine (triangles) were then added to the crude extract. Histidase activity was measured at a regular time for 4 days.

Table 2. Reactivation of EDTA-treated *D. tertiolecta* histidase

Metal ions	Activity (%)
Untreated enzyme	100
Enzyme with 50 mM EDTA	82.4
Mn^{2+}	123.6
Li^{2+}	105.1
Cu^{2+}	33.0
Fe^{2+}	44.3
Fe^{3+}	41.2
Co^{3+}	0

Crude extract was dialysed against EDTA 50 mM for 12 h before being incubated for 2 h in different metal solutions (10^{-4} M). Histidase activity was then recorded. The assays were run in triplicate.

cytosine derivatives were much more inhibitory than adenine or uracil derivatives. The degree of phosphorylation was relatively unimportant and nucleosides were as effective as nucleotides.

We investigated further the action of ATP, GTP and GMP. As chelation of an essential metal ion by the various nucleotides was considered initially as a possible explanation for the inhibition, studies were conducted in the presence of $MnCl_2$ and nucleotide effectors in excess. Because Mn^{2+}could not be increased above 0.1 mM at pH 9 without formation of insoluble $Mn(OH)_2$, some experiments were performed at pH 7.0 with Mn^{2+} present in at least a 2-fold excess over nucleotide. The effect of increasing concentration of ATP on the activity of histidase is given (Fig. 7). The results show that at 20 °C

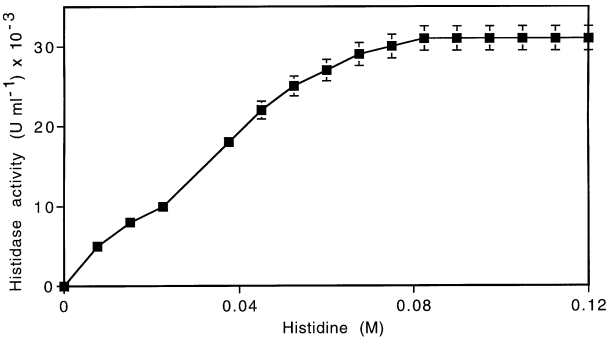

Fig. 3. Saturation curve of histidase versus histidine concentration.

Table 3. Kinetics parameters of purified histidase from *D. tertiolecta*.

	20 °C Histidine concentration < 22.5 mM	After heating > 22.5 mM	Any histidine concentration
V_m (U ml^{-1})	2×10^4	4.5×10^4	5.1×10^4
K_m (M)	2.25×10^{-2}	4×10^{-2}	1.3×10^{-2}
n	1	4	

The assays were run in triplicate.
V_m, enzyme maximal velocity; K_m, Michaelis constant; n, Hill coefficient.

ATP is an allosteric inhibitor of histidase. Similar results were obtained in the presence of GMP or GTP.

Discussion

This paper reports the purification and characterization of a histidine deaminating enzyme (histidase) from *Dunaliella tertiolecta*. Following IMAC chromatography, a yield of 51% was obtained, although it was only about 7.7% after DEAE chromatography. This result can be interpreted as the elimination of an inhibitor of histidase activity during the IMAC step.

The molecular mass of enzyme was close to 155 kDa estimated by gel filtration and 38.2 kDa when estimated by SDS-PAGE, indicating that the enzyme is a tetramer apparently containing four identical subunits of 38.2 kDa each. The native molecular mass (tetramer) is smaller than that found in *Pseudomonas aeruginosa* (198 kDa), *Bacillus subtilis* (220 kDa) and *Streptomyces griseus* (212 kDa) (Wu *et al.*, 1992).

The substrate saturation curves of histidase at room temperature display a biphasic behaviour, and two sets of Hill numbers and V_m and K_m values were determined when the histidine concentrations were higher or lower

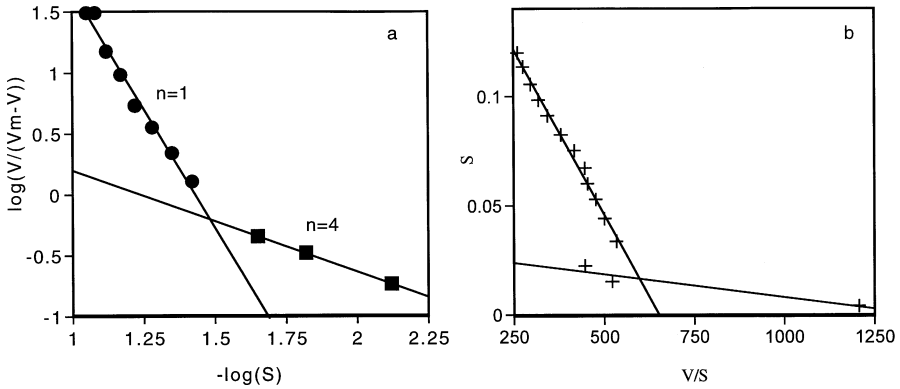

Fig. 4. (*a*) Hanes-Wolf plot of histidase saturation curves versus histidine concentration. (*b*) Hill plot representation.

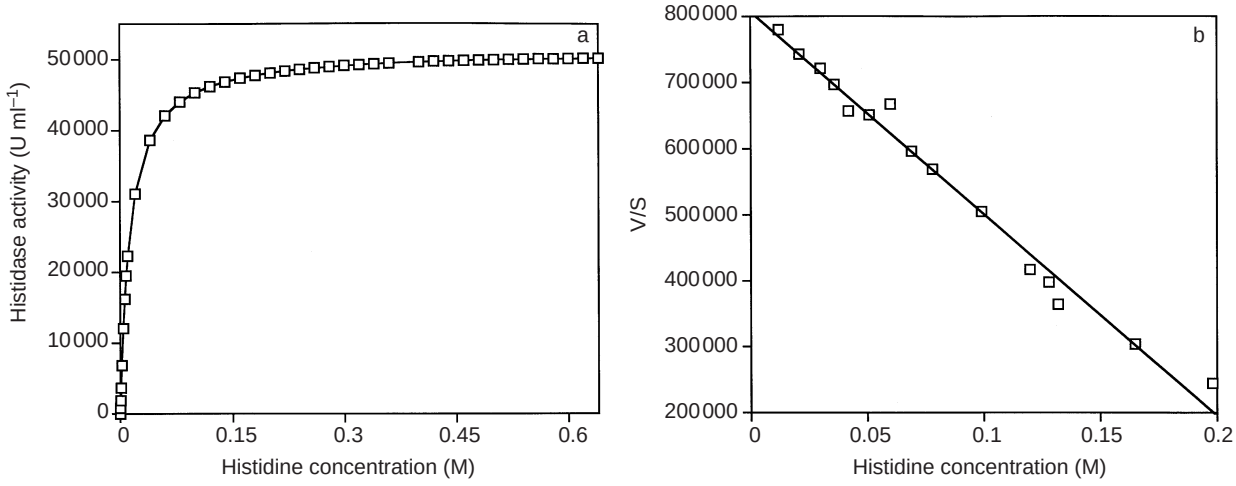

Fig. 5. Histidase versus histidine concentration after a heating step (10 min, 35 °C). (*a*) Saturation curve of histidase. (*b*) Hanes-Wolf plot representation of histidase saturation curve versus histidine concentration.

Table 4. Effects of Mn^{2+}, histidinol phosphate and L-histidine on cyanide inhibition of histidase.

Mn^{2+}, 5 mM	Histidinol phosphate, 50 mM	L-histidine 5 mM	Potassium cyanide 50 mM	Histidase activity after 2 h
−	−	−	−	82.5
−	+	−	+	51.5
−	+ 10 min incubation	−	+	82.5
−	−	+	+	51.5
−	−	+ 10 min incubation	+	82.5
+	−	−	+	51.5
+ 10 min incubation	−	−	+	75.2

Purified histidase was combined with potassium cyanide, L-histidine, histidinol phosphate and Mn^{2+} at the indicated concentration in trisaminomethane buffer (pH 8.8) in a total volume of 20 ml. Mixture was incubated for 2 h before measuring histidase activity. Results are presented as the percentage of activity in function of the activity at time $T = 0$ without cyanide.

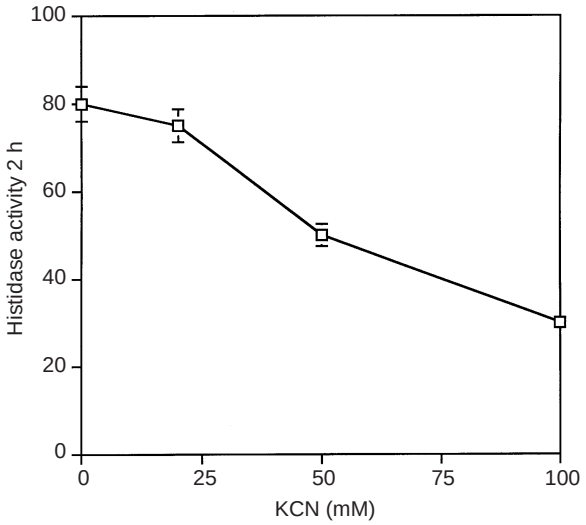

Fig. 6. Inactivation of histidase by cyanide (KCN). Purified histidase was combined with cyanide in trisaminomethane buffer (pH 8.8) in a total volume of 20 ml. Mixture was incubated for 2 h before measuring histidase activity as a function of activity at time $T = 0$ without cyanide.

Table 5. Specificity of the inhibition by effector

Effector	Histidase activity at 20 °C (%)
Glutamate 0.5 mM	100
Glutamine 0.5 mM	101
GTP 0.5 mM	24
GPD 0.5 mM	20
GMP	14
XMP 0.5 mM	13
XTP 0.5 mM	15
CTP 0.5 mM	28
CDP 0.5 mM	26
CMP 0.5 mM	20
UTP 0.5 mM	81
UDP 0.5 mM	78
UMP 0.5 mM	80
ATP 0.5 mM	82
ADP 0.5 mM	88
AMP 0.5 mM	89

Activity of purified histidase was measured, at 18 °C or after a heating step (10 min, 35 °C), in the presence of effector. Activity in the control was measured with 10 ml of purified histidase and 66 mM histidine. All activity measurements were done with 66 mM histidine. Each effector was dissolved in distilled water, pH was adjusted to 9.0, and effector was added to histidase at a final concentration of 0.5 mM. Results are expressed in comparison with the activity in the control.

than 22.5 mM respectively. When histidine concentration was lower than 22.5 mM, the Hill number (n) is 1 and when histidine concentration is higher than 22.5 mM, n is approximately 4. This is an indication that histidase may be allosterically regulated and show a cooperative response with respect to substrate concentration. This result has also been observed in *Pseudomonas aeruginosa* and in *P. testosteroni*, where the activity of the histidase at low concentrations of l-histidine is low (Wu *et al*., 1992).

This observation suggests that the histidase of *Dunaliella tertiolecta*, in addition to being regulated at the expression level, is also subject to more direct regulation operating at the level of enzyme activity. In the case of *P. testosteroni* (Coote & Hassal, 1973), it was suggested that histidine degradation in the cell had to be extremely low in order to prevent the breakdown of endogenous histidine. In the absence of a well-structured separation between degradative and biosynthetic activity in the cell, histidase may be allosterically regulated and display a cooperative response with respect to histidine concentration. Thus, there will be little activity at low concentrations at which histidine has to be recycled for the purpose of internal metabolic adjustments. Over a critical threshold (22.5 mM), the enzyme system becomes much more active in order to meet the needs of the cell for nitrogen. But if the same experiment is conducted following a heating step (1 h, 40 °C), the kinetics of histidase appear to follow a classical Michaelis-Menten profile. This simple experiment shows that a heating step may hide the allosteric character of histidase. Wu *et al*. (1992) found that all histidase preparations that did not show allosteric behaviour had been subjected to a heating step during purification.

Internal concentrations of histidine in microalgae varied from 5 to 50 mM in different studies (Turpin & Harrison, 1978; Admiraal *et al*., 1986; Martin-Jézéquel *et al*., 1988).

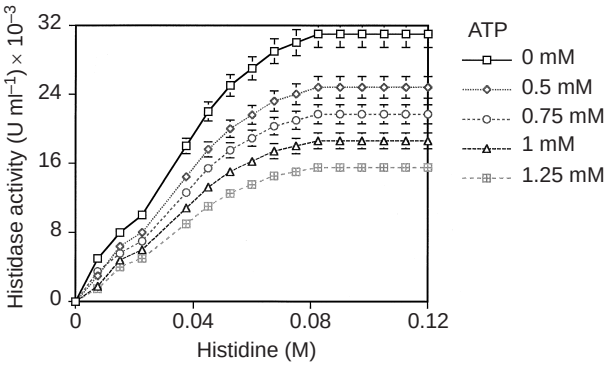

Fig. 7. Saturation curve of purified histidase at different ATP concentrations (0 mM to 1.25 mM).

These values were for the total cell volume and do not take into account any system of concentration or compartmentalization. These figures are clearly consistent with the K_m values determined for *Dunaliella tertiolecta* histidase. Most examinations of internal amino acid pools in algae have been performed using ODS columns with methanol as the organic solvent (Flynn, 1988). More recently Flynn & Flynn (1992) developed a new system and showed that the previous method of analysis could have confused histidine with another compound which, by this second method, co-elutes with glutamine. If estimates of internal histidine concentrations were lowered by a factor of 10, they would even remain consistent with amino acids functioning as substrates in the concentration range of 5×10^{-3} to 0.5 M.

Different mechanisms can modulate histidase activity both *in vitro* and *in vivo*. One possibility is that histidase can undergo a reversible change in conformation dependent on the phase of culture growth. MacClard & Kolenbrander (1973) have shown that histidase purified from *Pseudomonas fluorescens* comprises two conformational isomers that can be interconverted by temperature; no evidence was presented, however, to indicate that this mechanism regulated histidase activity *in vivo*. Another hypothesis is that histidase inactivation may occur by reversible polymerization, as Rechler (1969) suggested for histidase purified from *Pseudomonas* strain 11299. The polymerized forms of histidase were less active than the monomeric form.

The effects of thiol reagents on histidase were tested. Mercaptoethanol had no effect on histidase activity, contrary to dithiothreitol which had a positive effect in conserving histidase activity. The presence of dithiothreitol in the extraction buffer kept the enzymatic activity at its maximum level, although cysteine strongly inhibited histidase activity; these contrary effects of different thiols on histidase activity must be noted. Upon storage in the absence of thiols, histidase became oxidized to a less active form. The same reaction has been observed in *Pseudomonas* (Rechler, 1968). This reaction presumably involves disulphide bridge formation since thiols restore activity in a few minutes to an hour depending on the conditions. The possibility that some enzyme molecules are inactive on oxidation and some are not may be discounted, since the change does not appear to involve the active site directly. The presence of substrate does not block the restoration of activity and the catalytic properties of the enzyme are modified on oxidation. Thiols are used in enzyme assays in order that all the enzyme is in its reduced form; however, thiols inhibit as well as activate the enzyme. The optimal activity was greater with dithiothreitol than with mercaptoethanol, so the enzyme must have been incompletely reduced in the mercaptoethanol-containing assay. Thiols with a sulphydryl group adjacent to an amino group, such as cysteine, are better inhibitors than simple thiols.

Histidase from *Dunaliella tertiolecta* was not inhibited by metal-chelating agents such as EDTA. Mn^{2+}, the only metal ion among a large group tested which stimulated enzyme activity, did so by only 20%. The same result was found by Rechler (1969) in *Pseudomonas*. The stimulation of activity in the presence of Mn^{2+} can be explained by the fact that the inhibition by thiols is the direct result of coordination to a metal ion at the active site.

Several studies have pointed to an allosteric control of histidase but most have had difficulty in identifying a physiologically important regulator of histidase activity. The current findings provide a good indication that histidase may be allosterically regulated by some nucleotides, most notably purine nucleotides but possibly by cytosine nucleotides. Preliminary indications are that the inhibition by a mixture of effectors is simply additive. Thus, control of histidase would be achieved by the total concentration of all inhibitory compounds, each influenced by its binding constant. From a physiological viewpoint, however, the most likely regulators would be ATP and GTP, since these are in higher concentrations than any other nucleotides. Reversal of inhibition would require a drop in the pools of all inhibitory nucleotides and not simply a change in the degree of phosphorylation. The pattern of inhibition presently observed may reflect a biosynthetic aspect of the enzyme's function rather than its more acknowledged role in degradation for energy production via glutamate formation. One might reason that the histidine degradation pathway is an important source of the C_1 units essential for purine formation, since formyl glutamate, an intermediate in histidine degradation, can donate a formyl group to tetrahydrofolate (Silverman, 1962). Under conditions where histidine serves as a major carbon source, histidase may be regulated by the size of the total nucleotide pool, reflecting the need for the additional synthesis of purine nucleotides.

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