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Variations in Intracellular Choline Levels May Account for Differences in Glycine Betaine Synthesis Between Conspecific Oyster Populations Responding to Hyperosmotic Stress

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ABSTRACT Oysters (*Crassostrea virginica*) from the Atlantic coast use large intracellular concentrations of glycine betaine as an osmolyte to respond to hyperosmotic stress. In conspecific oysters from the Chesapeake Bay, intracellular concentrations of glycine betaine are low and do not change in response to high salinity stress. One possibility to explain this difference is that the availability of choline, the precursor of glycine betaine, is different in the two groups of oysters. We have measured the intracellular concentration of choline in both groups of oysters following adaptation to both low and high salinity, using a high-performance liquid chromatographic protocol. The choline concentrations in gill tissue from Atlantic and Bay oysters are similar following low salinity acclimation and similar to Bay oysters in the field. However, following high salinity acclimation, the intracellular choline pool increases substantially in Atlantic oysters, while declining slightly in Bay oysters. In addition to these results, we present a detailed protocol for measuring choline in tissues of marine organisms, where the analysis is complicated by the presence of high salts and free amino acids. *J. Exp. Zool. 278:283–289, 1997.*

Cell volume regulation in response to osmotic stress mediated by the regulation of small molecular weight organic molecules is a well-known phenomenon. Over the years, this regulatory mechanism has been studied in a wide array of species. The majority of these studies have focused on the volume regulatory mechanisms which utilize intracellular free amino acids, even though the older literature indicates that many species use other organic osmolytes (Pierce, '82). In particular, the quarternary ammonium compound glycine betaine was often mentioned in these early studies (Bricteux-Gregoire et al., '62, '64). Until recently, subsequent investigators, including us, paid little further attention to the quarternary ammonium compounds. However, it has become apparent that some very euryhaline species, including oysters (*Crassostrea virginica*), have relatively small amino acid pools and utilize quarternary ammonium compounds in addition to the amino acid osmolytes (Warren and Pierce, '82; Pierce et al., '84, '92). In particular, glycine betaine is turning out to be a routinely encountered osmolyte in many extremely osmotically tolerant bacteria (reviewed in Le Rudulier and Bernard, '86), plants (Flowers et al., '77; Storey and Wyn Jones, '77; Pan et al., '81), and animals (Warren and Pierce, '82; Yancey et al., '82), including mammals (Bagnasco et al., '86). While the mechanism of glycine betaine synthesis regulation in response to hyperosmotic stress has been studied in some detail in plant and microbial species, culminating in the discovery of the remarkable osmogene system of osmoregulation in bacteria (Strom et al., '86) and spinach leaf cells (Weretilnyk and Hanson, '89), almost nothing is known about the regulation of glycine betaine synthesis in animals except for our previous work on *Limulus* (Dragolovich and Pierce, '94) and oysters (Pierce et al., '92, '95).

We have been examining the mechanism(s) controlling salinity-induced glycine betaine synthesis in *C. virginica* for two main reasons. First, the oyster is a fairly euryhaline, osmoconforming species that uses a combination of intracellular free amino acids and glycine betaine for cell volume regulation (Pierce et al., '92). Second, oysters from the Chesapeake Bay (Bay oysters,

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hereafter) and their Atlantic conspecifics (Atlantic oysters, hereafter) differ significantly in their intracellular response to hyperosmotic stress. Gill tissue from Bay oysters contains a very low level of glycine betaine, which does not fluctuate when the animals are exposed to higher salinities. In contrast, gills from Atlantic coast individuals, taken from oyster populations along the U.S. East Coast (Florida to Cape Cod), have at least tenfold higher amounts of glycine betaine compared to Bay oysters, and this level increases twofold following adaptation to high salinity. Interestingly, Bay oysters are much less salinity-tolerant than Atlantic animals, likely a result of the lower glycine betaine levels (Pierce et al., '92).

Choline is the precursor of glycine betaine everywhere it has been tested, including in oysters. Isolated gill mitochondria from both oyster populations take up choline and convert it to glycine betaine. The rates of mitochondrial choline uptake are similar over a wide range of choline concentrations (20 μM–10 mM) in both oyster populations acclimated to low salinity. In mitochondria taken from oysters adapted to high salinity, choline uptake differs between the two populations only at very high concentrations of substrate (10 mM). At choline concentrations of 5 mM or less, the mitochondria from high salinity adapted Bay oysters take up choline at the same rate as Atlantic mitochondria and synthesize similar amounts of glycine betaine (Pierce et al., '95). Thus, although there are some differences in the choline uptake kinetics between the two oyster populations, it is clear that following adaptation to high salinity, isolated Bay oyster mitochondria can take up choline and convert it to glycine betaine in a manner similar to the high salinity adapted Atlantic conspecifics. These results suggest that, unless intracellular choline concentrations are very high (>5 mM), choline uptake is not the source of difference in glycine betaine accumulation in the two oyster populations. However, differences in choline availability between the two oyster groups might account for the variation in glycine betaine accumulation patterns.

We have measured intracellular choline in gill tissue from Bay and Atlantic oysters acclimated to both low and high salinity, as well as in gill tissue from Bay oysters in the field. Our results indicate that while the choline concentration is similar in gill tissue from low salinity adapted oysters from both populations, it increases substantially only in high salinity adapted Atlantic oysters.

MATERIALS AND METHODS

Choline concentrations in gill tissue from Atlantic and Bay oysters (*C. virginica*) were measured using a high-performance liquid chromatographic (HPLC) method modified from several protocols designed for such measurements in mammalian tissues (for example, Potter et al., '83; Tyrefors and Carlsson, '90; Murray et al., '90; Ikarashi and Maruyama, '93; Fossati et al., '94; Shen et al., '95). Both the high salt and free amino acid content of oyster tissue (and of marine organisms, in general) interfere with the analysis and require the addition of several steps to the protocol. The method we used is described below first, followed by some caveats regarding its use.

Animal acclimation

Atlantic oysters, purchased from Mook Sea Farm (Walpole, ME) and Bay oysters, collected from the Choptank River in the Chesapeake Bay, were acclimated to artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio) of either 350 or 950 mosm. Acclimation schedules differed depending on the acclimation salinity and the oyster population. Both Atlantic and Bay oysters were directly transferred to low salinity and allowed to acclimate for at least 2 weeks. However, unlike the Atlantic oysters, which were directly transferred to 950 mosm and allowed to acclimate for at least 4 weeks, the less salinity-tolerant Bay oysters were initially acclimated to 750 mosm for 4 weeks prior to the 4 weeks of acclimation in 950 mosm (Pierce et al., '92). All the oysters were starved during the acclimation period.

Extraction of tissue choline

Gills were excised from the acclimated oysters, immediately frozen on dry ice, and lyophilized. In addition, in order to determine whether or not starvation affects gill choline concentrations, gills were removed from several Bay oysters as they were collected in the field (255 mosm at the time of collection). The field-excised gills were frozen on dry ice immediately and transported back to the laboratory, where they were lyophilized. Dry weights were determined and several gills pooled to a total dry weight of 235 mg/sample. Gills were homogenized in 40% ethanol, and the homogenate was boiled for 10 min to precipitate protein and then centrifuged at 20,000*g* for 15 min at 4°C. The resulting supernatant was frozen, lyophilized, and dissolved in 2 ml of double distilled water (ddH_2O). This solution was applied to an ion retardation column (1.1 cm × 45 cm, AG11A8, 50–

100 mesh; Biorad, Richmond, CA), and choline was eluted with ddH_2O at a flow rate of 1.5 ml/min. Based on preliminary runs that measured the elution of ^{14}C-choline, the first 14 ml of eluant were discarded and the next 20 ml collected as a single fraction, frozen, lyophilized, and redissolved in ddH_2O. This chromatography removed approximately 40% of the total inorganic salts present in the samples.

Next, the choline (and any other quaternary amines) was separated from most of the remaining salts and amino acids by reineckate precipitation (Warren and Pierce, '82). Choline was precipitated from the redissolved eluent by the addition of an equal volume of freshly made, filtered, ice-cold reineckate solution (2%) and acidified by the addition of HCl (final concentration 0.5 N). The samples were then vortexed and incubated on ice for 2 hr. Following incubation, the samples were centrifuged at 12,000*g* for 30 min at 4°C. The resulting supernatant was discarded and the pellet, containing the choline, resuspended in ddH_2O. An equal volume of ethyl acetate was added to remove any remaining reineckate. Samples were vortexed and the organic and aqueous phases separated by centrifugation (1,000*g*, 15 min). The aqueous phase containing the choline was retained and extracted twice more with ethyl acetate. After the final extraction, a known volume of the aqueous phase was transferred to a clean Erlenmeyer flask and placed under a stream of N_2 to remove any traces of ethyl acetate, then frozen and lyophilized.

Choline measurement

Choline concentrations in the extracts were measured using an isocratic HPLC procedure. Lyophilized, reineckate-treated samples were resuspended in 500 µl of mobile phase (100 mM $NaPO_4$, 7 mM trimethylamine [TMA], pH 7.6, using phosphoric acid), filtered (0.2 µm) (Gelman, Ann Arbor, MI), and injected onto a cation exchange analytical column (Hamilton PRP-X200, 0.5 × 25 cm; Phenomenex, Torrance, CA) of the HPLC (model 421; Beckman, Fullerton, CA), equipped with a precolumn pulse damper and a precolumn filter. Choline was separated with the mobile phase described above (flow rate = 0.6 ml/min) and detected electrochemically after passing through a postcolumn reactor containing choline oxidase (C-5896; Sigma, St. Louis, MO) conjugated to cyanogen bromide (CNBr)-activated sepharose 4B (Pharmacia, Gaithersburg, MI) (Damsma et al., '85), where it was converted to glycine betaine and hydrogen peroxide. The H_2O_2 peak was identified by an electrochemical detector (BAS, LC-4A, dual platinum working electrode) set at a potential of +500 mV and quantified by a computer (Shimadzu C-R1A). Ethylhomocholine bromide (EHCB) was used as an internal standard as well as a control to monitor the efficacy of the choline oxidase activity in the postcolumn reactor. EHCB was prepared as previously described (Potter et al., '83; Eva et al., '84). Briefly, equal volumes of 8.45 M dimethyl-3-amino-1-propanol (Aldrich, Milwaukee, WI) and 13.4 M bromoethane (Aldrich) were mixed and allowed to crystallize for 30 min at room temperature. Diethyl ether was added to the crystals and immediately decanted. Crystals were dried for 1 hr at room temperature and then dissolved in methanol with vigorous shaking. Ethyl acetate was added to the solution, one drop at time, until formation of the white precipitate stopped. The mixture was then capped and incubated overnight at 4°C. All liquid was removed by placing the solution first under a stream of N_2 for 1 hr, then under vacuum until a fine white powder formed (approximately 1 hr). The dry EHCB was stored at –20°C. EHCB was dissolved in the mobile phase and added to the samples just prior to HPLC analysis.

Finally, to verify that we were indeed measuring choline, portions of several processed samples were preincubated in choline oxidase (Sigma), which converts choline to glycine betaine, prior to HPLC analysis. Following choline oxidase treatment, the choline peak was not detected (Fig. 1).

Statistics

Intracellular choline concentrations were statistically compared using Student's *t*-test with a software package (Stat-view II). $P \leq 0.05$ was accepted as significant.

Methodological caveats

The protocol described above will adequately extract and quantify choline in tissues with high salt and amino acid contents. However, both the extraction and analytical procedures need to be done with more than a little attention to detail. The following points are of particular importance: (1) We processed all of our samples using the same two ion retardation columns. Columns were packed on different days but with the same batch of resin. Both adequately desalted the samples but with different recoveries (76% ± 6 and 59% ± 4). Furthermore, the recoveries of choline from each column were slightly different between the oyster populations at each salinity, likely due to the

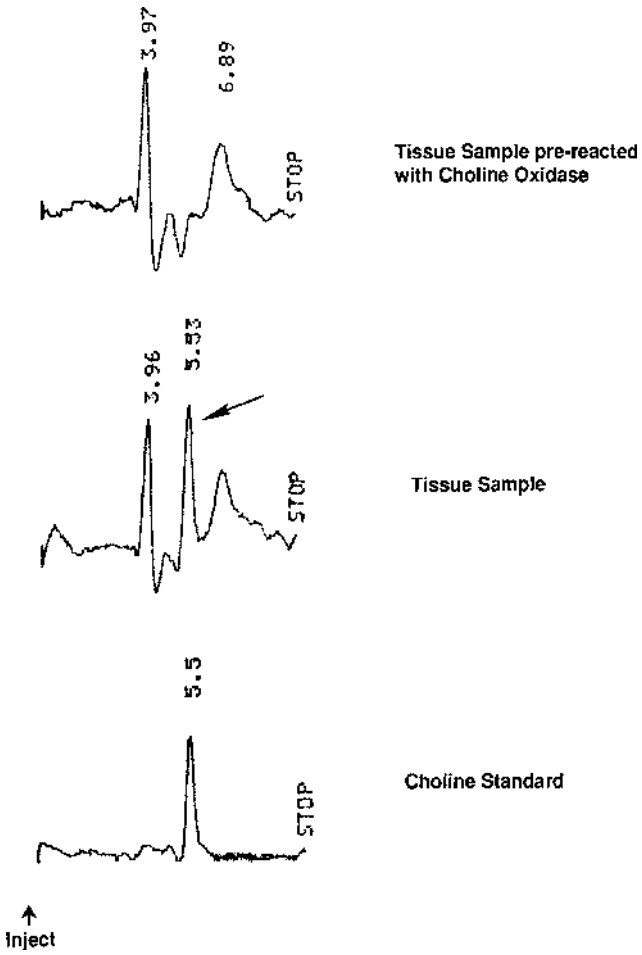

Fig. 1. HPLC chromatograms of choline standard (3 nmol, lower chromatogram), a tissue sample extracted according to the protocol described in the "Materials and Methods" (middle chromatogram), and the same tissue extract pretreated with choline oxidase (upper chromatogram). The choline peak (arrow, middle chromatogram) is entirely eliminated by choline oxidase treatment (compare middle and upper chromatograms). The numbers above each chromatogram indicate time (min) from the injection of sample onto the HPLC column.

amount of choline in the samples. Thus, we ran recovery samples routinely with each sample batch on each of the ion retardation columns. In addition, after every ten samples, the elution profile of each column was checked by running a ^{14}C-choline standard while monitoring both the radioactivity and osmolality of the eluent. Final choline values were corrected for the recovery from the ion retardation column, the recovery from the oyster tissue batch, and the recovery from the reineckate precipitation step (Warren and Pierce, '82). Final recoveries of choline from the entire extraction procedure were either 59% or 46%, depending upon which ion retardation column was used.

(2) Column care is also critical. Between samples, we found it was necessary to wash the columns with a minimum of eight bed volumes ddH_2O to insure that all of the salts from the previous sample were removed. In addition, after every six to eight samples, the columns required regeneration with one bed volume of 0.5 M NaCl to maintain adequate performance. The top 1 cm of each column was replaced after the elution of 12–16 samples, followed by regeneration with 0.5 M NaCl. Finally, if the columns were not used for several days, they were stored in 0.03% NaAzide at room temperature. Removal of the azide required a wash with 1 l ddH_2O and then regeneration with one bed volume of 0.5 M NaCl followed by a 500 ml ddH_2O rinse.

(3) Some of the early studies that led to the development of the HPLC protocol described above used silica matrix cation exchange analytical columns, such as Nucleosil 5 SA (Damsma et al., '85). When coupled with the enzyme reactor-electrochemical detection protocol, ion exchange (as opposed to reverse-phase) chromatography is the best choice for choline analysis because the mobile phase is compatible with choline oxidase activity in the postcolumn reactor, but the required pH causes rapid and irreversible failure of the analytical column matrix. Some of our columns became completely blocked in less than a day. Although more expensive, the broader pH tolerance of the polymer matrix in the Hamilton PRP-X200 cation exchange column provides long column life at a pH compatible with both choline separation and enzyme reactor activity (Haen et al., '91).

(4) Postcolumn reactor enzyme activity decreased with both time and number of samples run through it. This gradual decrease in sensitivity needs to be compensated for by the inclusion of an internal standard and fairly frequent standard curve recalibrations. Typically, we ran a choline standard every three or four samples to monitor the postcolumn enzyme activity. Eventually, the enzyme reactor had to be replaced, usually after 35–40 tissue samples or 100 standard choline samples had been run.

(5) Finally, as is usually the case with electrochemical detection, electrode stability and sensitivity required lengthy equilibration with the mobile phase and, once attained, was best maintained by continuous flow. Nevertheless, electrode quality deteriorated with time. Inclusion of the internal standard helped to compensate for the

deterioration, but it was important to occasionally disassemble the flow cell and clean the electrodes. Furthermore, we found that briefly reversing the electrode polarity at the end of the day (Damsma et al., '85) resulted in significantly improved chromatography the next day.

RESULTS

Intracellular choline concentration was not significantly different in Bay and Atlantic oyster gills following acclimation to 350 mosm (Fig. 2). The low salinity acclimated Atlantic oyster gills contained 18.2×10^2 nmol/g dry weight (± 2.7) compared to 15.2×10^2 nmol/g dry weight (± 2.0) in the Bay gills from the same salinity. Furthermore, the choline content of the low salinity acclimated oysters was statistically indistinguishable from that in the gills of Bay oysters sampled in the field (11.4×10^2 nmol/g dry weight ± 2.1). On the other hand, the intracellular choline concentrations were quite different in the gills of the two oyster populations following acclimation to high salinity. In the Atlantic gills, the choline concentrations increased more than 2.5-fold, to 45.9×10^2 nmol/g dry weight (± 9.5), while the concentrations in the Bay oyster gills actually declined to 6.1×10^2 nmol/g dry weight (± 0.8) (Fig. 2).

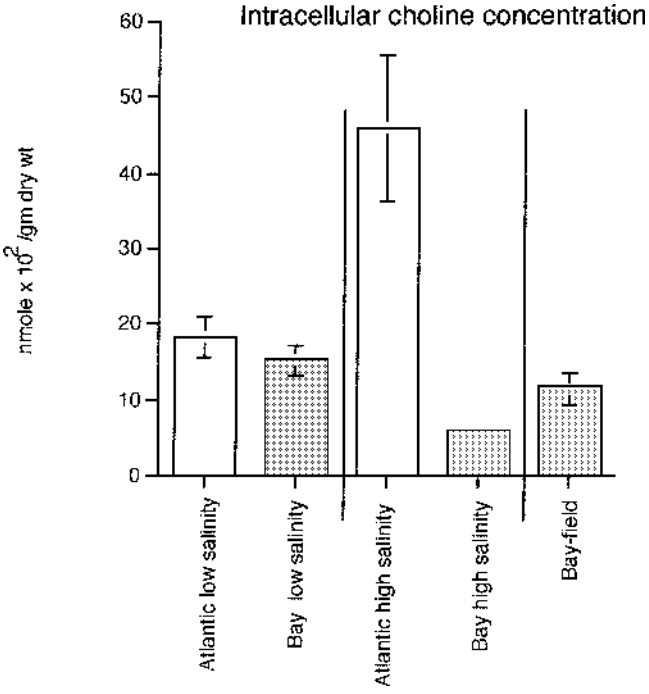

Fig. 2. A comparison of mean intracellular choline concentrations in gills from Atlantic oysters (open bars) and Bay oysters (shaded bars) following acclimation to either high (950 mosm) or low (350 mosm) salinity. Sample sizes were as follows: Atlantic high salinity, four samples from nine animals; Atlantic low salinity, nine samples from 20 animals; Bay high salinity, 14 samples from 36 animals; Bay low salinity, 14 samples from 46 animals. Also shown is the concentration of intracellular choline in gills of Bay oysters, sampled in the field (255 mosm). The sample size for the field group was eight samples from 27 oysters. Error bars indicate standard error of the mean (SEM). The SEM for the Bay high salinity data was less than the width of the border.

DISCUSSION

The gill cells of Atlantic oysters accumulate choline during acclimation to a hyperosmotic stress. In contrast, choline concentration in Bay oyster gills declines during acclimation. These population differences in choline concentration may account for the differences in glycine betaine accumulation in the two oyster groups in response to high salinity (Pierce et al., '92). In addition, the choline differences may point to the presence of control mechanisms of glycine betaine synthesis upstream from choline.

Possibly, intracellular choline levels are the rate-limiting factor in glycine betaine synthesis. In this case, the increased intracellular choline concentrations that occur in high salinity adapted Atlantic oysters would result in increased glycine betaine production and subsequent elevation of the intracellular concentration of that osmolyte. In the Bay oysters, on the other hand, glycine betaine concentrations would not increase for lack of precursor, and as we have shown elsewhere, they do not (Pierce et al., '92, '95). Furthermore, since we starved all of the experimental oysters, the increased choline in the Atlantic oyster gills indicate the presence of a mechanism to synthesize choline, which the Bay oysters lack or which was not activated by the high salinity stress. Thus, these results suggest that, in addition to the biochemical steps leading from choline to glycine betaine, the production of choline may be involved in the regulation of glycine betaine synthesis by oysters in response to hyperosmotic stress. These results need to be interpreted with care because it is likely that all of the Bay oysters were parasitized with the haplosporidium protist *Perkinsus marinus* (Dermo) (Paynter et al., '95). Very few biochemical consequences of *P. marinus* are presently known (Paynter, '96), so at this point it is impossible to determine whether the differences in choline metabolism between the two oyster groups are a result of genetics or some parasite-induced effect.

Although a wide variety of plants are capable of choline biosynthesis, usually by the step-wise

methylation of ethanolamine (Weretilnyk et al., '95; Hanson and Rhodes, '83; Greenberg, '69), choline is often a dietary requirement in many animal species. Furthermore, very little is known about choline biosynthesis in animals other than humans, rats, and agriculturally important domestic species. Other than in the *Loligo pealei* optic nerve (Anfuso et al., '95), nothing is known about choline biosynthesis among molluscan species. In animal cells (including *Loligo*) where choline synthesis occurs and has been studied, it takes place through a series of phosphatide base intermediates. The precursor of the phosphatide bases is serine, which is decarboxylated to ethanolamine. The methylations occur at the level of these phospholipid-bound bases to produce phosphatidylcholine, which is subsequently converted to free choline (Datko et al., '90; Hanson and Rhodes, '83; Greenberg, '69). Serine, a glycolytically produced amino acid, is also the precursor of glycine. Since intracellular glycine concentrations increase very rapidly in intact Bay oysters exposed to hyperosmotic stress (Paynter et al., '95), perforce, both glycolysis (at least to the level of 3-phosphoglycerate) and serine production must be rapidly increased in response to hyperosmotic stress in both oyster populations. Thus, the difference in the regulation of glycine betaine production between the two oyster groups might reside in the steps between serine and choline in addition to the terminal enzyme of the pathway to glycine betaine, as we have reported earlier (Perrino and Pierce, '95). We are presently evaluating these new possibilities experimentally.

Finally, in spite of its metabolic importance, the difficulty in analyzing for choline seems to have prevented the accumulation of much information about it, especially in nonmammalian species. Although the use of postcolumn enzyme reactors and electrochemical detector-equipped HPLCs has helped to make choline analysis specific and more routine, the preparation of choline samples for this type of analysis from the tissues of marine animals is still difficult. The high intracellular concentrations of salts and amino acids in marine organisms interfere with the electrochemical detection of choline and are not easily removed from choline in cleanup procedures. The protocol we used in the experiments reported here adequately lowers the inorganic salt concentrations and removes the amino acids, but the isolation procedure, the enzyme reactor column, and the electrochemical detector all require vigorous and constant attention to detail in order to reliably quantify tissue choline levels.

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