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Choline Dehydrogenase Kinetics Contribute to Glycine Betaine Regulation Differences in Chesapeake Bay and Atlantic Oysters

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ABSTRACT Choline dehydrogenase (CD), the first enzyme of the glycine betaine synthetic pathway, was measured in a mitochondrial lysate from gill tissue from Atlantic and Chesapeake Bay oysters acclimated to both 350 and 750 mosm. CD from both populations functions at its maximum rate at 30°C and pH 8.75. Although CD from both populations has a similar affinity for its substrate, choline (K_m = 15.7 mM), CD V_{max} from Atlantic oysters is twice that from Bay oysters. In addition, the CD K_m doubles and the V_{max} increases four-fold in both oyster populations acclimated to 750 mosm. CD activity is competitively inhibited by both betaine aldehyde and glycine betaine. The differences in CD kinetics between the two oyster populations help to account for the lower glycine betaine synthesis rates and concentrations in Chesapeake Bay oysters. CD cannot function rapidly enough to saturate the enzyme, betaine aldehyde dehydrogenase (BADH), immediately downstream, and, therefore, CD kinetics limit the rate of glycine betaine synthesis in oysters. *J. Exp. Zool. 286:250–261, 2000.*

Cell types from different species capable of withstanding a broad range of external osmotic concentrations accumulate a variety of osmolytes in an effort to counteract the resulting movement of water. Glycine betaine is an intracellular osmolyte that accumulates in the cells of a wide range of salinity-tolerant organisms, including bacteria (Perroud and LeRudulier, '85; Tombras-Smith et al., '88; Falkenberg and Strom, '89; Mori et al., '92), mammalian kidney (Haubrich and Gerber, '81; Grossman and Hebert, '89; Garcia-Perez and Burg, '91), plants (Storey and Wyn Jones, '77; Hanson and Scott, '80; Hanson et al., '85; Weigel et al., '86; Weretilnyk and Hanson, '89; Wood et al., '96), and a number of marine invertebrates (Pierce et al., '92; Dragolovich, '94), such as the eastern oyster, *Crassostrea virginica*, in response to hyperosmotic stress. However, glycine betaine concentrations in gill tissue from both low- and high-salinity–acclimated Chesapeake Bay oysters are significantly lower than those in Atlantic conspecifics. Furthermore, while glycine betaine concentrations increase in Atlantic oysters under hyperosmotic conditions, they do not increase in Chesapeake Bay oysters (Pierce et al., '92).

In most cell types studied to date, glycine betaine is the product of a two-step oxidation of choline. The first reaction (choline → betaine aldehyde) is catalyzed by choline dehydrogenase (CD) and the second (betaine aldehyde → glycine betaine) is catalyzed by betaine aldehyde dehydrogenase (BADH). In animal cell types, including oyster gill cells, the entire glycine betaine synthetic pathway is located within the mitochondria (Dragolovich, '94; Pierce et al., '95). However, the regulation of this pathway is not well understood in animal cells. In *Limulus* cardiac cells, choline uptake into the mitochondria is the limiting step in glycine betaine synthesis (Dragolovich, '94). In contrast, while isolated mitochondria from both Atlantic and Bay oyster populations acclimated to low salinity take up choline at similar, linear rates over a very wide choline concentration range, Atlantic oyster mitochondria subsequently synthesize more glycine betaine (Pierce et al., '95). Therefore, differences in the glycine betaine synthetic pathway following mitochondrial choline uptake must exist between the two populations.

We have shown elsewhere that BADH kinetic differences partly account for the differences in glycine betaine synthesis rates between the two

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oyster populations acclimated to low salinity (Perrino and Pierce, '99). However, the 10-fold glycine betaine concentration differences between the two populations in the whole acclimated animals (Pierce et al., '92) are probably not explained by the three-fold BADH substrate affinity differences alone, indicating that CD kinetic differences might also exist between the two populations. In addition, because the kinetics governing the accumulation of the BADH substrate, betaine aldehyde, are unknown, whether the BADH kinetic differences alone could account for the entire difference in glycine betaine synthesis is unclear. Oyster BADH has a high substrate affinity and low saturating substrate concentration, which corresponds well with a regulatory role. As a result, only relatively low concentrations of substrate are needed to produce the enzyme's faster reaction rates and to reach saturation. Thus, glycine betaine synthesis rates would be limited at fairly low substrate levels. However, isolated oyster mitochondrial glycine betaine synthesis rates increase over a very wide range of choline concentrations (Pierce et al., '95), well above the betaine aldehyde saturating concentration of BADH (Perrino and Pierce, '99). Thus, the CD reaction may not produce sufficient concentrations of betaine aldehyde that would saturate BADH. If this is true, CD would have a role in limiting the rate of glycine betaine synthesis as well.

In addition, both oyster populations contain similar intracellular choline concentrations at low salinity (Pierce et al., '97). However, the choline level in Bay oysters acclimated to high salinity does not increase from that in low salinity, while the choline levels in high-salinity–acclimated Atlantic oysters increase four-fold (Pierce et al., '97). Examination of CD kinetics would also help to determine if the differences in choline availability at high salinity limit CD product formation, producing the glycine betaine differences between the two populations.

The source of choline in oysters is not known. While diet is a possibility, tissue choline increases in hyperosmotically stressed, starved oysters, suggesting that the choline source is at least in part synthetic (Pierce et al., '97). Almost nothing is known about choline biosynthesis in molluscs except in the *Loligo pealei* optic nerve, where choline originates from serine (Alberghina and Gould, '90; Anfuso et al., '95), which is a glycolytically produced amino acid (Stryer, '81). In a number of organisms, including a variety of plants, dogfish neural tissue and rat neurons, as well as *Loligo* optic nerve, synthesis then proceeds through ethanolamine and a series of phosphatide base intermediates (Hanson and Scott, '80; Hanson and Rhodes, '83; Andriamampandry et al., '89; Alberghina and Gould, '90; Datko et al., '90; Weretilnyk et al., '95). Thus, the control of glycolysis, which is also affected in bivalves by salinity changes (Baginski and Pierce, '78), could influence substrate availability for glycine betaine synthesis.

Therefore, we tested the possibility that CD kinetics are different between the two populations, limiting the rate of synthesis of glycine betaine in oysters. We determined that differences exist in CD kinetics between the two oyster populations and that the kinetics are altered following acclimation to high salinity in both populations. The results suggest that because of a very low substrate affinity for choline and a modest reaction rate, CD conversion of choline to betaine aldehyde should limit betaine aldehyde accumulation and, therefore, BADH activity and subsequent glycine betaine synthesis in both populations.

MATERIALS AND METHODS

Animals

Crassostrea virginica from the Chesapeake Bay were purchased from commercial seafood companies on Maryland's eastern shore. *Crassostrea virginica* from the Atlantic coast were obtained from Mook Sea Farms in Damariscotta, ME, whose oysters were grown in the Damariscotta River in southern Maine. All of the oysters were acclimated to 350 mosm artificial seawater (Instant Ocean) and 10°C for a minimum of 2 weeks, the amount of time required for bivalves to acclimate to low salinity (Pierce, '70). High-salinity oysters were acclimated to 750 mosm artificial seawater at 10°C for at least 4 weeks prior to use, the minimum amount of time required for bivalves to acclimate to high salinity (Baginski and Pierce, '77).

Isolation of mitochondria

Since the complete glycine betaine synthetic pathway resides in the oyster gill mitochondria (Pierce et al., '95), a mitochondrial fraction was prepared using the method of Pierce et al. ('95). Approximately 10 g of freshly dissected gill tissue was minced and homogenized in 10 volumes (wt:vol) of mitochondrial isolation buffer (MIB) containing 0.4 M sucrose, 150 mM mannitol, 20 mM HEPES-KOH, 1 mM ethylene glycol-bis-

N,N,N′,N′-tetraacetic acid (EGTA), pH 7.5. For the high-salinity experiments, the osmolality of the isolation buffer was adjusted with an additional 250 mM mannitol. The tissue was homogenized with a motor-driven Teflon homogenizer (Tri-R, Model S63C). A total of 10 passes were made at a motor setting of 3.5. The homogenate was diluted to approximately 2 liters with isolation buffer and passed three times through a double layer of Miracloth (Calbiochem, San Diego, CA). The filtrate was then centrifuged at 2,000*g* for 15 min (SLA-1500, Sorvall, Wilmington, DE) to remove large cellular debris. The supernatant was centrifuged at 9,000*g* for 25 min. The resulting pellet was resuspended in a small volume of isolation buffer and briefly rehomogenized. This suspension was then centrifuged for 15 min at 2,000*g* (SS-34, Sorvall), after which the supernatant was centrifuged for 20 min at 8,500*g*. The pellet from the last centrifugation step, which contained the mitochondria (Pierce et al., '95), was resuspended, and this set of centrifugation steps was repeated once more in a smaller volume of isolation buffer. All isolation procedures were carried out at 0–4°C. The mitochondrial pellet from the second set of centrifugation steps was resuspended in a reaction buffer containing 50 mM HEPES (pH 8.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 21 μM leupeptin, 0.5 mM phenylmethylsulfonylfluoride (PMSF, added slowly as a 200-mM stock in isopropanol), and 0.1% Triton X-100 (v/v) to lyse the mitochondria (Perrino and Pierce, '99). The suspension was then centrifuged at 6,600*g* for 5 min. The pellet was discarded and the supernatant used to assay CD activity.

CD assay

Mitochondrial protein (0.4–0.5 mg), ^{14}C-choline chloride (specific activity = 0.2 μCi/μmol) (Amersham) labeled at the methyl carbons, were combined with varying concentrations of nonradioactive choline chloride (5–80 mM) and the electron acceptor, 1 mM phenazine methosulfate (PMS), (Grossman and Hebert, '89; Zhang et al., '92) in the following reaction:

$$\underset{\text{choline}}{(CH_3)_3\text{-N-}CH_2\text{-}CH_2\text{-OH}} + \text{PMS} \xrightarrow{-2H^+} \underset{\text{betaine aldehyde}}{(CH_3)_3\text{-N-}CH_2\text{-CHO}} + \text{PMS (reduced)}$$

The assay mixture was designed to measure the conversion of choline to betaine aldehyde and any potential conversion of betaine aldehyde to glycine betaine (see below). CD activity was dependent on the presence of PMS. Maximum CD activity occurred at a PMS concentration of 1 mM, which was used throughout the CD experiments. PMS concentrations over 1 mM inhibited CD activity.

CD activity was measured in mitochondria lysed with Triton X-100 to prevent choline uptake across the mitochondrial membranes from limiting the reaction rates. The reaction was started with the addition of the mitochondrial lysate and then incubated at 28°C in the dark to prevent the light-induced reduction of PMS. Preliminary experiments using nonsaturating choline concentrations indicated that Triton X-100 did not interfere with the assay.

After 60–120 min of incubation—the amount of time determined in preliminary experiments to produce measurable and linear amounts of reaction products using this amount of protein—the reaction was stopped with the addition of one tenth of the reaction volume of 1.2 N HCl. The reaction mixtures were then divided in half and 0.007–0.009 μCi ^{14}C-glycine betaine and ^{14}C-betaine aldehyde (the amount determined in preliminary experiments) added to one of the two halves of each sample to determine recovery efficiency. The spiked and unspiked sample halves were then processed identically as described below.

Twice the reaction volume of chloroform and one reaction volume of methanol were added to the samples. The samples were vortexed and centrifuged briefly to separate the aqueous and organic phases (Zhang et al., '92). A portion of the upper methanol phase was removed and dried under nitrogen. The dried residues were then resuspended in the mobile-phase HPLC buffer described below. The samples were filtered through a syringe filter (0.2 μm pore size) and 100 μl injected onto a normal-phase HPLC column (Phenomenex, Phenosphere, 5 μm, silica, 150 × 3.2 mm). The reaction products, betaine aldehyde and glycine betaine, and the unreacted choline were separated isocratically (Beckman model 334) with a mobile phase consisting of 800 ml acetonitrile, 64 ml methanol, 5 ml 3:2 (v/v) 1.0 M ammonium acetate/acetic acid, 127 ml water, and 10 ml 0.1 M KH_2PO_4 at a flow rate of 1.3 ml/min (modified from Zhang et al., '92). The eluent from the column was collected in 15- to 30-sec fractions into scintillation vials and the radioactivity determined by liquid scintillation counting (LKB Rackbeta) to determine which fractions contained betaine aldehyde, as well as glycine betaine, the result of any subsequent betaine aldehyde oxidation. The rate of product formation was calculated as nmol prod-

ucts (betaine aldehyde and glycine betaine) produced/min/mg protein. Protein was determined by a modified Lowry assay (Petersen, '77) using bovine serum albumin as a standard.

Control experiments

Because CD kinetics were significantly different between low- and high-salinity–acclimated oysters in both populations, control experiments were conducted to ensure that the source of these differences was not the MIBs. Low-salinity–acclimated oyster mitochondria were isolated as described above, but in high salinity, MIB and the CD assay performed as usual.

There were no differences in CD activity between the low-salinity–acclimated mitochondria isolated in low-salinity MIB and the low-salinity mitochondria isolated in high-salinity MIB in either oyster population, indicating that the CD kinetic differences between low and high salinity were not a result of the MIBs used.

Preparation of ^{14}C-betaine aldehyde

Although ^{14}C-glycine betaine was available (a gift from Dr. Maurice Burg, National Institutes of Health, Bethesda, MD), no commercial sources of radiolabeled betaine aldehyde were available. Therefore, ^{14}C-betaine aldehyde was synthesized using choline oxidase from bacterial *Alcaligenes* species, which catalyzes the conversion of choline to glycine betaine through a betaine aldehyde intermediate (Ikuta et al., '77; Zhang et al., '92). Choline oxidase (0.008 units, Sigma Chemical Company, St. Louis, MO) was incubated in the CD reaction buffer described above with 0.4 μCi ^{14}C-choline chloride and 12.0 mM unlabeled choline chloride at 37°C for 10–40 min. The reactions were stopped at varying times and the reaction products extracted and analyzed by HPLC as described above. Over the course of the reaction, three radioactive peaks were identified and collected from the HPLC, two corresponding to the retention times of choline and glycine betaine and a third that was subsequently identified as betaine aldehyde by thin layer chromatography (TLC) (see below). As the reaction was allowed to proceed, the choline peak diminished while the amount of glycine betaine increased. The size of the betaine aldehyde peak remained fairly constant over the course of the reaction until the end, where all the original radioactivity was present in the glycine betaine peak. Once the optimum reaction time to produce the most labeled betaine aldehyde was determined, the betaine aldehyde peak from a number of these choline oxidase reactions was collected and the HPLC buffer removed under a stream of nitrogen. This pool of dried ^{14}C-betaine aldehyde was resuspended in a small volume of water and frozen until use. The ^{14}C-betaine aldehyde and ^{14}C-glycine betaine were then used to determine product recovery in the mitochondrial CD assays.

Verification of reaction products by TLC

TLC was used in order to confirm the identity of the radiolabeled peaks separated by HPLC from both the *Alcaligenes* choline oxidase reaction and the oyster mitochondrial CD reaction. The fractions containing the 3 radiolabeled peaks were each dried under a stream of nitrogen and dissolved in 50% ethanol. The dissolved material was streaked on precoated silica plates (LK5D, Whatman, Clifton, NJ) and developed consecutively three times in a one-dimensional system of chloroform: methanol: 0.1 M HCl (65: 30: 4; v/v) (modified from Zhang et al., '92). Individual standards of choline, betaine aldehyde, glycine betaine, and trimethylamine (TMA), another possible oxidation product of choline, were chromatographed in parallel. At the end of the third run, the plates were thoroughly air-dried and the quaternary ammonium standards visualized with Dragendorff's reagent (Rowland and Pierce, '85). The region of the TLC plate that corresponded to the migration distances of the standards was scraped off from both the bacterial choline oxidase and the oyster mitochondrial CD sample lanes. The radioactivity of the scraped silica was determined by liquid scintillation counting (LKB Rackbeta). The radioactivity in each of the three peaks from both reactions corresponded to the R_f of the choline, betaine aldehyde, or glycine betaine standards.

Because the TLC separation between betaine aldehyde and TMA was minimal, the dried putative betaine aldehyde peaks from both *Alcaligenes* and oyster mitochondrial reactions were oxidized in 25% H_2O_2 and 1.0 N NaOH at room temperature to oxidize any betaine aldehyde to glycine betaine (Grossman and Hebert, '89). The oxidized samples were redried under nitrogen, streaked, and developed on the silica TLC plate as described above. All of the radioactivity in both oxidized bacterial and mitochondrial samples was present in the spot corresponding to glycine betaine, indicating that the original peak was, in fact, all betaine aldehyde.

Statistical analysis

The results were tested for significance using analysis of variance (ANOVA) with the Tukey-Kramer post hoc test for unequal n following ANOVA (Hinkle et al., '98) and Student's *t*-tests for a planned comparison (Hinkle et al., '98). A probability of $P < 0.05$ was considered significant.

RESULTS

The HPLC protocol described in the Materials and Methods section successfully separated choline from its two oxidation products, betaine aldehyde and glycine betaine. Choline oxidase from *Alcaligenes* oxidizes choline to glycine betaine through a betaine aldehyde intermediate (Ikuta et al., '77). As the *Alcaligenes* choline oxidase reaction proceeded, betaine aldehyde concentrations tended to remain fairly constant. The amount of choline decreased steadily, apparently producing betaine aldehyde at a rate similar to betaine aldehyde's conversion to the end product, glycine betaine, which increased over the course of the reaction (Fig. 1A–C).

Maximum CD activity for both low- and high-salinity–acclimated oysters from both populations occurred at 28–30°C and pH 8.75 (adjusted at 28°C), although Atlantic maximum rate occurs over a pH range that is somewhat broader (Fig. 2A, B). Therefore, all reactions were run at 28°C and pH 8.75. CD product formation was linear over the entire 2-hr reaction time used in initial experiments, ensuring an accurate rate calculation.

CD activity was dependent on the presence of an electron acceptor, PMS. No activity was observed without the electron acceptor. Concentrations of PMS above 1–2.0 mM inhibited CD activity. CD from low-salinity–acclimated oysters from both populations had a K_m of 15.7 mM (±1.5) for its substrate, choline. CD from Atlantic oysters had a higher V_{max} of 3.9 nmol/min/mg protein (±0.4), two times higher than that from Bay oysters (Fig. 3), indicating that CD from Atlantic oysters functions faster at all choline concentrations.

In both oyster populations, both betaine aldehyde and glycine betaine competitively inhibited CD activity to a similar degree. The V_{max} remained the same, but the K_m increased to 86.6 mM (±25) when 60 mM betaine aldehyde was present and to 55.4 mM (±6.7) with an additional 60 mM glycine betaine in Atlantic oysters (Fig. 4). In Bay oysters, the K_m increased to 71.0 mM (±25) in the presence of 60 mM betaine aldehyde and to 35.3 mM (±13) in the presence of 60 mM glycine betaine (Fig. 5). CD from high-salinity–acclimated oysters had significantly higher maximum velocities than those from low-salinity–acclimated oysters. The V_{max} for CD from high-salinity Atlantic oysters was 11.6 nmol/min/mg protein, three-fold higher than that from low-salinity oysters (Fig. 6). The V_{max} for CD from high-salinity–acclimated Bay oysters was 6.5 nmol/min/mg protein, also three-fold higher than in low salinity (Fig. 7). As was the case with the low-salinity enzymes, the V_{max} for high-salinity–acclimated Bay oyster CD was two times lower than the V_{max} from high-sa-

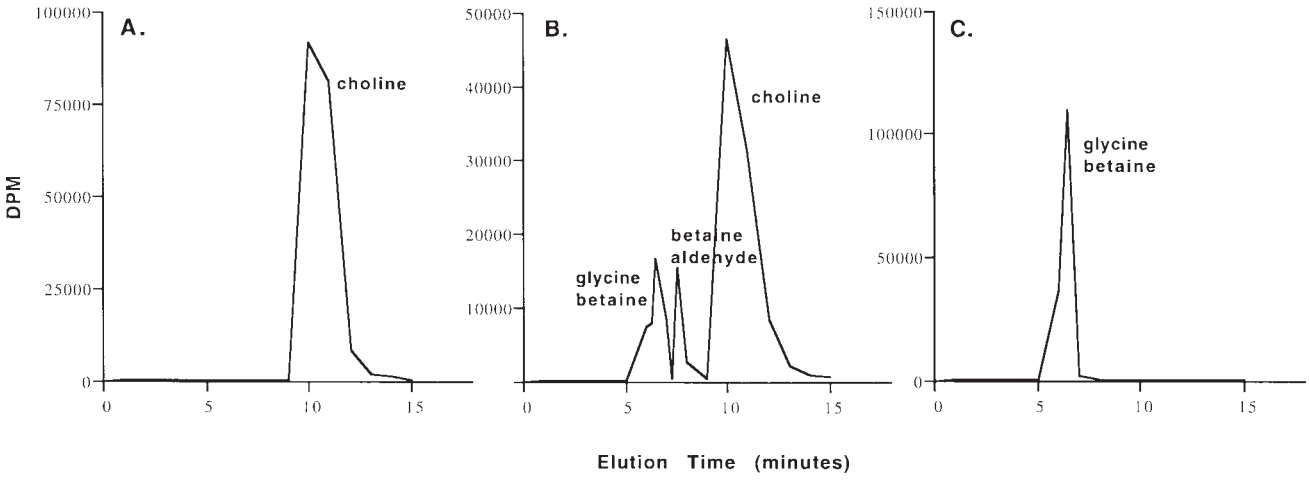

Fig. 1. Chromatograms of the separation of the products of the *Alcaligenes* choline oxidase reaction when ^{14}C-choline is provided as substrate. **A:** Reaction time = 0, only ^{14}C-choline, the starting material, eluted from the HPLC column. **B:** Reaction time = 12 min, a combination of the reaction intermediate, ^{14}C-betaine aldehyde, and of the reaction end product, ^{14}C-glycine betaine, as well as some of the original ^{14}C-choline is present. **C:** Reaction time = 24 min, only the end product, ^{14}C-glycine betaine, remains.

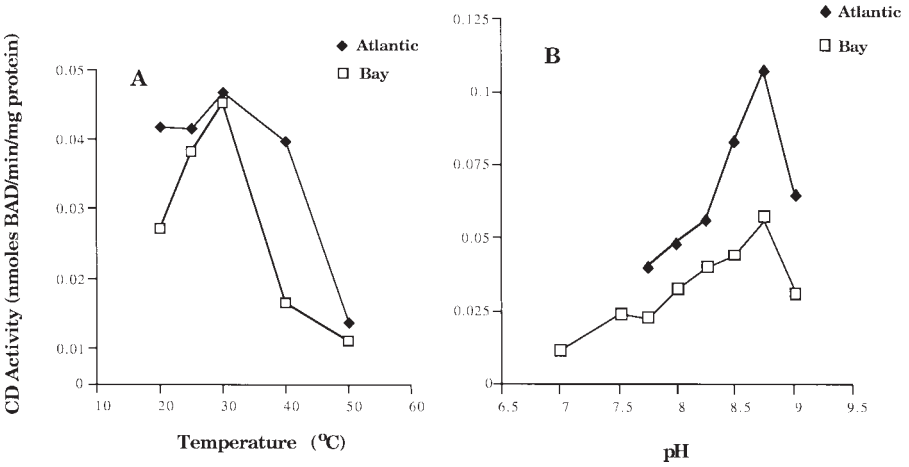

Fig. 2. **A:** The effect of temperature on oyster CD activity. **B:** The effect of pH on CD activity.

linity–acclimated Atlantic oysters (Fig. 8). In addition, the K_m also increased to 31.3 mM (±2.9) in high-salinity–acclimated oysters from both populations (Figs. 6–8).

DISCUSSION

CD is localized in the mitochondria of both oyster populations acclimated to low and high salinity. Although CD from both Atlantic and Bay oysters acclimated to low salinity has a similar K_m for choline, the higher V_{max} in Atlantic oysters adds to the explanation of the population differences in glycine betaine levels and synthesis rates. The lower CD rate in Bay oysters, which produces the substrate for BADH, together with the lower BADH substrate affinity must result in a lowered glycine betaine production compared with Atlantic oysters. The greater substrate affinity of Atlantic oyster BADH means that it is better able to convert the higher concentrations of betaine al-

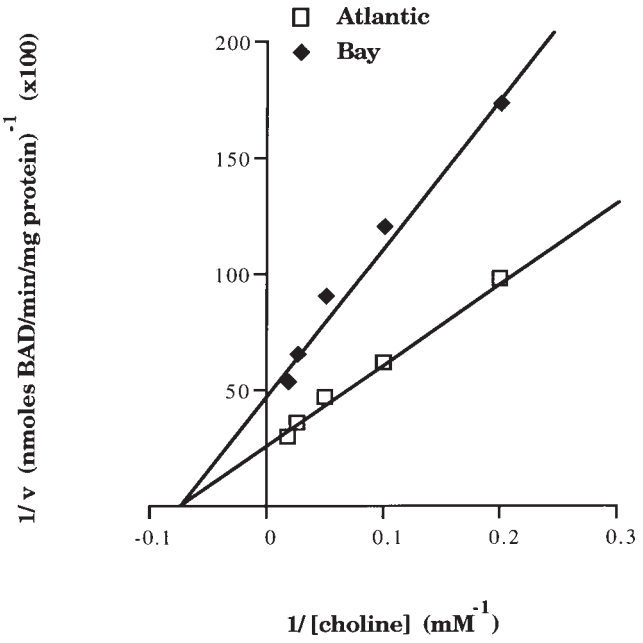

Fig. 3. Double reciprocal plot of CD activity in a mitochondrial lysate from Atlantic oysters (V_{max} = 3.9 nmol/min/mg protein) and Bay oysters (V_{max} = 1.8 nmol/min/mg protein) acclimated to 350 mosm as a function of varying concentrations of the substrate, choline. The K_m for both reactions was 15.7 mM choline.

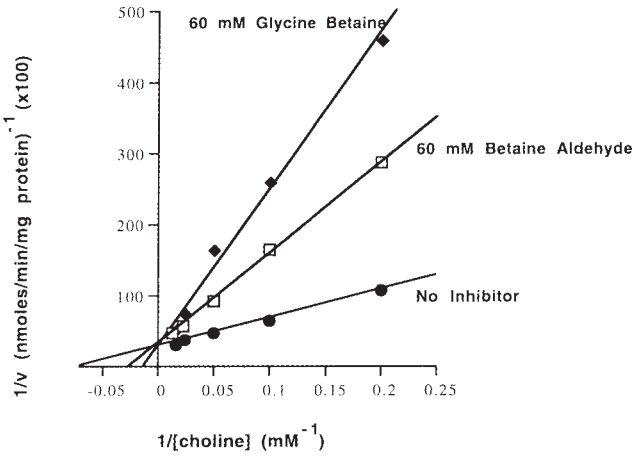

Fig. 4. Double reciprocal plot of the activity of CD in a mitochondrial lysate from Atlantic oysters acclimated to 350 mosm as a function of varying concentrations of the substrate, choline. The effects of the competitive inhibitors, betaine aldehyde (K_i = 24.0 mM) and glycine betaine (K_i = 33.0 mM) are also shown. The V_{max} for all reactions was 3.9 nmol/min/mg protein.

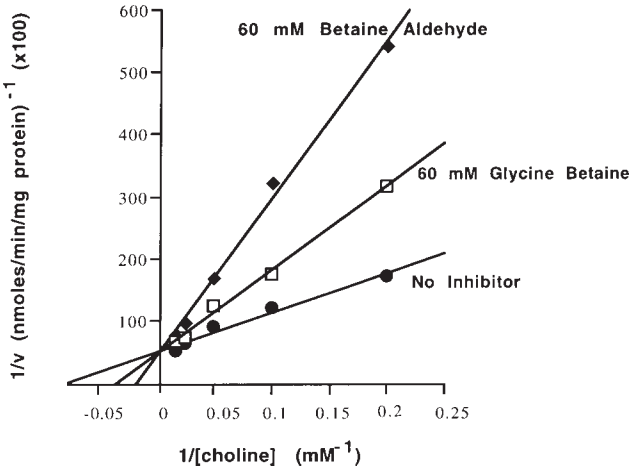

Fig. 5. Double reciprocal plot of the activity of CD in a mitochondrial lysate from Bay oysters acclimated to 350 mosm as a function of varying concentrations of the substrate, choline. The effects of betaine aldehyde (K_i = 70.5 mM) and glycine betaine (K_i = 21.4 mM), competitive inhibitors, are also shown. The V_{max} for all reactions was 1.8 nmol/min/mg protein.

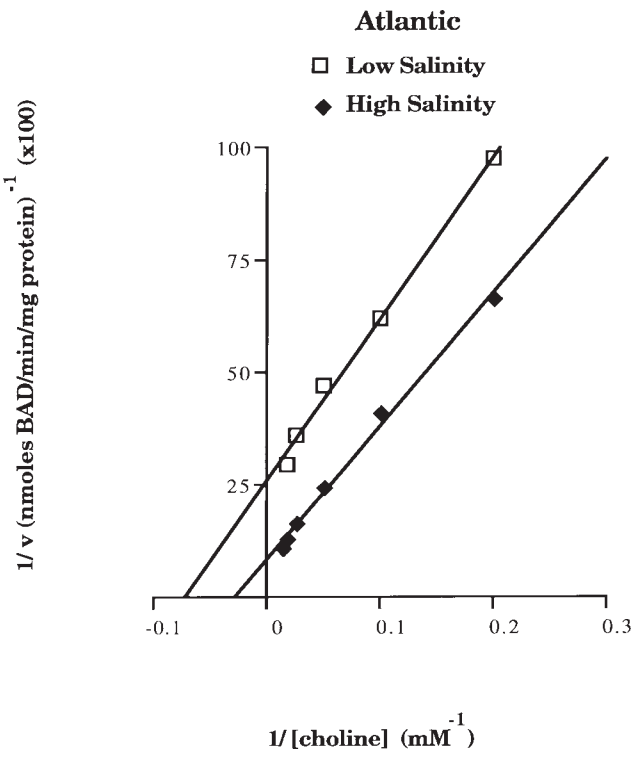

Fig. 6. Double reciprocal plot of CD activity in a mitochondrial lysate from Atlantic oysters acclimated to 350 mosm (K_m = 15.7 mM choline, V_{max} = 3.9 nmol/min/mg protein) and to 750 mosm (K_m = 31.3 mM choline, V_{max} = 11.6 nmol/min/mg protein) as a function of varying concentrations of the substrate, choline.

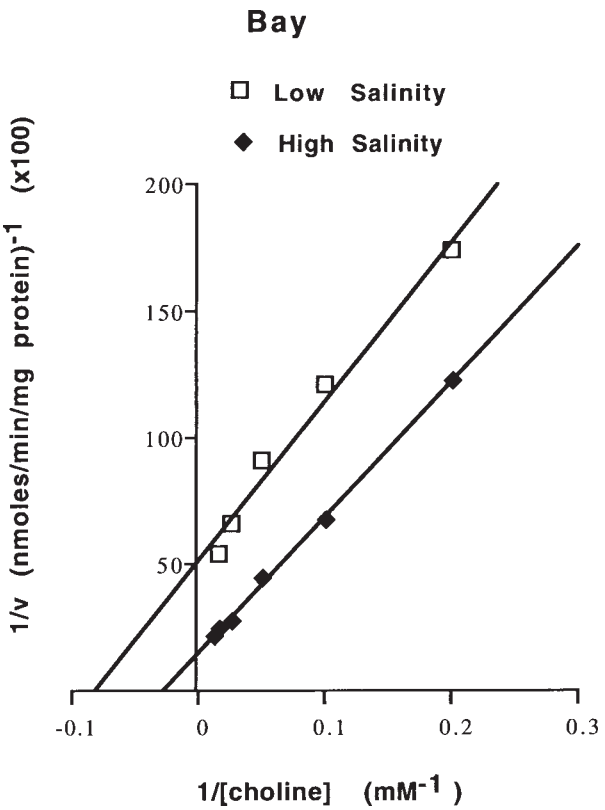

Fig. 7. Double reciprocal plot of CD activity in a mitochondrial lysate from Bay oysters acclimated to 350 mosm (K_m = 15.7 mM choline, V_{max} = 1.8 nmol/min/mg protein) and 750 mosm (K_m = 31.3 mM choline, V_{max} = 6.5 nmol/min/mg protein) as a function of varying the substrate, choline.

dehyde, produced by the higher Atlantic CD rate, to glycine betaine.

There are two possible explanations for the increased CD reaction rates in high-salinity–acclimated oysters. The first is that something associated with high salinity increases the specific activity of the enzyme. The increase in CD reaction velocity in high-salinity–acclimated oysters from both populations should lead to increased betaine aldehyde concentrations and, subsequently, increased rates of glycine betaine synthesis. The increase in CD reaction rate in high salinity is similar to the increase in BADH activity during acclimation of spinach to 300 mM NaCl (Weigel et al., '86; Weretilnyk and Hanson, '89) and to that in *Rhizobium meliloti* and *E. coli* when grown in high osmolality (Landfald and Strom, '86; Tombras-Smith et al., '88). In addition, the activity of CD also increases four-fold in *E. coli* when grown in high salt medium (Landfald and Strom, '86). The other possible explanation for the increased CD rates is that the amount of enzyme increases during high-salinity acclimation.

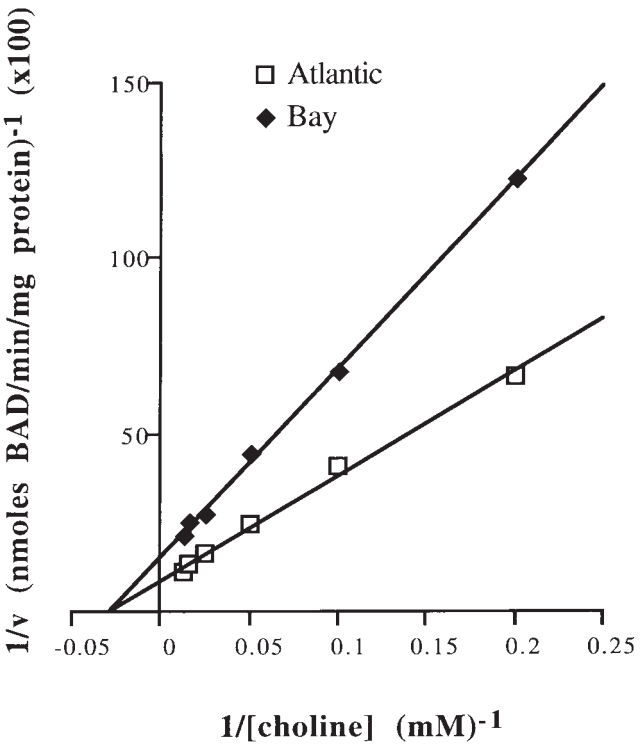

Fig. 8. Double reciprocal plot of the activity of CD in a mitochondrial lysate from Atlantic oysters (V_{max} = 11.6 nmol/min/mg protein) and Bay oysters (V_{max} = 6.2 nmol/min/mg protein) acclimated to 750 mosm as a function of varying concentrations of the substrate, choline. The K_m for both reactions was 31.3 mM choline.

In this case, the CD-specific activity might not change, while the increased amount of enzyme is able to synthesize more product. This possibility would require increased transcription or translation of the CD gene, analogous to osmogene function in bacteria and plants (Landfald and Strom, '86; Tombras-Smith et al., '88; Weretilnyk and Hanson, '89). Distinguishing between these possibilities here is difficult because if the increase in the amount of CD was quite small in comparison to the total amount of protein in the mitochondrial lysate, only an increase in CD-specific activity might seem to occur.

In addition to the increase in CD rate, the K_m also increases to 30 mM in high-salinity–acclimated oysters from both populations, which should slow the rate of betaine aldehyde synthesis and, ultimately, the rate of glycine betaine synthesis if choline concentrations remain constant. Possibly, choline concentrations transiently increase substantially, above the 0.25–1.0 mM measured in high-salinity–acclimated Bay and Atlantic animals (Pierce et al., '97), immediately following hyperosmotic stress. If a transient increase in choline levels occurred, a decrease in the CD substrate affinity would not slow the rate of betaine aldehyde production while the choline levels were elevated. Afterward, the choline levels might then decline to the levels measured in the acclimated animals. In fact, an analogous transient accumulation of alanine occurs following hyperosmotic stress in oysters and other bivalves (Baginski and Pierce, '77; Bishop et al., '94; Paynter et al., '95). Alanine, whose carbon source is glycolytic (Baginski and Pierce, '78; Bishop et al., '94), rapidly accumulates, reaching high levels within a few hours following hyperosmotic stress. As acclimation proceeds, alanine levels fall and are replaced by other free amino acids (Baginski and Pierce, '77; Bishop et al., '94; Paynter et al., '95). The elevation of choline concentrations soon after the onset of hyperosmotic stress, followed by a subsequent decline as acclimation proceeds seems to be a reasonable possibility since glycine betaine concentrations are up to 80-fold greater (Pierce et al., '92) than choline concentrations in acclimated whole animals (Pierce et al., '97). Although we have not yet measured it in oysters, glycine betaine concentrations increase substantially in *Limulus* cardiac tissue (Dragolovich and Pierce, '91) soon after the onset of hyperosmotic stress. In any event, it would be difficult to achieve such large increases in glycine betaine concentration in the oyster tissue without a substantial increase in choline levels during acclimation.

The K_m of oyster CD is quite high, which suggests several interesting points about its role in glycine betaine regulation in oysters. The K_m of oyster CD for choline is an order of magnitude higher than that of oyster BADH for betaine aldehyde (Perrino and Pierce, '99). Thus, CD should function considerably more slowly than BADH. This also occurs in *E. coli* where CD activity is lower than the other steps of the pathway and apparently limits the rate of glycine betaine synthesis (Landfald and Strom, '86). The CD K_m is also much higher than the measured tissue choline concentrations, a further indication that CD functions well below its maximum rate in acclimated whole animals. In addition, the high CD K_m suggests that the rate of choline oxidation by the mitochondria in the whole animal would depend to a large extent upon the regulation of the amount of free choline present in the tissue and on the rate of subsequent mitochondrial choline uptake. Tissue choline levels must be regulated either in the synthesis steps between serine and

choline or by glycolysis, since serine is a glycolytic amino acid (Stryer, '81). Indeed, glycolysis is stimulated by high-salinity stress in bivalves (Baginski and Pierce, '78).

In addition, large increases in tissue choline concentration should be reduced by the glycine betaine pathway, since CD activity continues to increase over such a wide range of choline concentrations, at least until mitochondrial uptake becomes limiting. This may be reflected by the relatively low tissue choline concentrations, 0.25–1.0 mM (Pierce et al., '97), in comparison to the higher tissue glycine betaine concentrations, 5–80 mM, in acclimated animals (Pierce et al., '92). Increases in choline concentration would be consumed by the glycine betaine pathway and result in the low choline levels and substantially greater glycine betaine concentrations.

CD from both oyster populations is competitively inhibited by the products of the two reactions in the glycine betaine pathway, betaine aldehyde and glycine betaine. The glycine betaine produced is transported out of the mitochondria into the cytoplasm to increase intracellular osmotic pressure. Therefore, intramitochondrial glycine betaine concentrations probably never get high enough to inhibit CD activity. BADH also has a much higher substrate affinity than CD, indicating that the betaine aldehyde produced in the CD reaction probably would not accumulate either but would be rapidly converted to glycine betaine. Even in Atlantic oysters, where betaine aldehyde is produced three-fold more quickly than in Bay oysters, because of the faster CD reaction rate, the three-fold greater BADH affinity for betaine aldehyde should still not permit betaine aldehyde accumulation to reach levels that would inhibit CD activity.

Although CD from lysed oyster gill mitochondria from both populations exhibited maximum activity at a lower temperature, 25–30°C, than that from mammalian kidney and liver (37°C; Haubrich and Gerber, '81; Grossman and Hebert, '89; Zhang et al., '92), the maximum activity of oyster CD occurs at a temperature that corresponds more appropriately with the temperature of this enzyme's environment most of the time. Unlike oyster gill BADH, oyster gill CD activity is much less limited by a high temperature for maximum activity under most conditions.

As is the case with CD from several other sources, oyster CD activity was dependent on the presence of an electron acceptor (Haubrich and Gerber, '81; Landfald and Strom, '86; Grossman and Hebert, '89; Zhang et al., '92). However, the identity of the endogenous mitochondrial electron acceptor is unknown. In rat liver mitochondria, CD activity is linked to ubiquinone in the electron transport chain (Barrett and Dawson, '75). Although CD kinetics were the same with PMS or ubiquinone in rat liver mitochondria (Barrett and Dawson, '75), the oyster gill CD kinetic parameters could be different with the natural electron acceptor. The inhibition of oyster CD by PMS concentrations above 1–2.0 mM also occurs in rat liver and kidney CD (Haubrich and Gerber, '81; Zhang et al., '92).

Overall, the kinetics of CD from oyster gill exhibit a slower conversion of choline to betaine aldehyde when compared with the kinetics of CD from several other sources (Table 1). Oyster gill CD V_{max} is somewhat lower (2–11.6 nmol/min/mg protein depending on the population and salinity) than that from rat liver (25.3 nmol/min/mg protein; Zhang et al., '92), rat kidney (37 nmol/min/mg protein; Grossman and Hebert, '89), *E. coli* (21–57 nmol/min/mg protein; Landfald and Strom, '86) and also lower than that from *Rhizobium* (71–145 nmol/min/mg protein; Tombras-Smith et al., '88). The K_m for CD from oyster gill (15.7–31.3 mM) is also higher than that from rat liver (0.14–0.27 mM; Zhang et al., '92), rat kidney (4.7 mM; Grossman and Hebert, '89), *E. coli* (1.5 mM; Landfald and Strom, '86), and *Rhizobium* (5.5 mM; Tombras-Smith et al., '88)

Finally, CD kinetic differences between the two populations could be explained in several ways. The primary structure of CD may vary slightly in each oyster population, conferring different kinetic characteristics to each. Since CD activity was measured in a mitochondrial lysate, some form of allosteric control of CD by another mitochondrial protein that differs between the two populations is possible. It is also likely that most of the Bay oysters were infected with the haplosporidean protozoan, *Perkinsus marinus,* given their size and age (Paynter and Burreson, '91). Although *P. marinus* infection significantly alters the whole animal's ability to respond to salinity stress (Paynter et al.,'95), it is unlikely that differences in CD kinetics in an isolated mitochondrial lysate would be due to the presence of this parasite since both the assay conditions and substrate levels were externally regulated. However, while CD differences found here are not likely a result of infection, the parasite might cause substrate or kinetic changes in the intact cells of the whole animal by altering the enzyme's environment. For

TABLE 1. Comparison of assay conditions for CD from several sources

Source	Preparation	Assay temperature (°C)	Assay pH (buffer)	Electron acceptor	K_m (mM)	V_{max} (nmol/min/mg protein)	Reference
Rhizobium meliloti	Cell extract	30	7.5 (80 mM sodium phosphate)	0.5 mM PMS	5.5	71–145	Tombras-Smith et al., '88
Escherichia coli	Cell extract or washed membranes	37	7.5 (80 mM sodium phosphate)	0.5 mM PMS	1.5	21–57	Landfald and Strom, '86
Rat inner medulla	Triton-extracted mitochondria	37	7.5 (50 mM sodium phosphate/50 mM Tris)	1 mM PMS	4.7	37	Grossman and Hebert, '89
Rat liver	Digitonin-extracted mitochondria	37	7.6–8.5 (40 mM Tris or glycine or 10 mM potassium phosphate)	1 mM PMS	0.14–0.27	6.3–25.3	Zhang et al., '92
Oyster gill	Triton-extracted mitochondria	30	8.75 (50 mM HEPES-KOH)	1 mM PMS	15.7–31.3	2–11.6	This paper

example, since *P. marinus* infection lowers hemolymph pH by 0.5–1.0 pH unit (Paynter, '96), CD activity may be decreased in the gill tissue of whole animals infected with *P. marinus*.

In conclusion, the major steps of the glycine betaine synthetic pathway in oysters have now been measured, and a more complete picture of the pathway can be described. In low salinity, since tissue choline availability and choline uptake by the isolated mitochondria are similar in both populations, a combination of a lower CD reaction rate and a lower BADH affinity for betaine aldehyde in Bay oysters probably explain both the glycine betaine concentration and synthesis rate differences between the two populations. The low CD affinity for choline and modest reaction rate also suggest that CD kinetics limit the rate of glycine betaine synthesis in oysters. The low CD substrate affinity indicates that the regulation of choline availability must ultimately regulate glycine betaine synthesis.

Finally, it is apparent that the basic glycine betaine synthetic machinery is in place in both oyster populations prior to osmotic stress. We cannot rule out changes in the amounts of enzymes associated with substrate synthesis, mitochondrial choline uptake, or glycine betaine synthesis, but the differences in enzyme kinetics between the two oyster populations seem to account reasonably well for the observed differences in glycine betaine recovery patterns. While a role played by osmogenes (Landfald and Strom, '86; Styrvold et al., '86; Eshoo, '88) is not yet apparent in the regulation, the differences between the two oyster populations in the enzymes suggest genetic differences between the populations.

LITERATURE CITED

Alberghina M, Gould RM. 1990. Levels of choline intermediates in the visual system structures and in peripheral nerve of the rat: comparison with neural tissues of a lower vertebrate (*Mustelus canis*) and an invertebrate (*Loligo pealei*). Neurochem Int 17:599–604.

Andriamampandry C, Freysz L, Kanfer JN, Dreyfus H, Massarelli R. 1989. Conversion of ethanolamine, monomethylethanolamine and dimethylethanolamine to choline-containing compounds by neurons in culture and by the rat brain. Biochem J 264:555–562.

Anfuso CD, Sipione S, Lupo G, Alberghina M. 1995. Evolutionary comparison of enzyme activities of phosphatidylcholine metabolism in the nervous system of an invertebrate (*Loligo pealei*), lower vertebrate (*Mustelus canis*) and the rat. Comp Biochem Physiol 112B:493–501.

Baginski RM, Pierce SK. 1977. The time course of intracellular free amino acid accumulation in tissues of *Modiolus demissus* during high salinity adaptation. Comp Biochem Physiol 57:407–412.

Baginski RM, Pierce SK. 1978. A comparison of amino acid accumulation during high salinity adaptation with anaerobic metabolism in the ribbed mussel, *Modiolus demissus demissus*. J Exp Zool 203:419–428.

Barrett MC, Dawson AP. 1975. The reaction of choline dehydrogenase with some electron acceptors. Biochem J 151:677–683.

Bishop SH, Greenwalt DE, Kapper MA, Paynter KT, Ellis LE. 1994. Metabolic regulation of proline, glycine, and alanine accumulation as intracellular osmolytes in ribbed mussel gill tissue. J Exp Zool 268:151–161.

Datko AH, Aksamit RR, Mudd SH. 1990. Phosphatidylcholine synthesis in the rat: the substrate for methylation and regulation by choline. Lipids 25:135–142.

Dragolovich J. 1994. Dealing with salt stress in animal cells: the role and regulation of glycine betaine concentrations. J Exp Zool 268:139–144.

Dragolovich J, Pierce SK. 1991. Comparative time course of inorganic and organic osmolyte accumulation as horseshoe crabs (*Limulus polyphemus*) adapt to high salinity. Comp Biochem Physiol 102A:79–84.

Eshoo M. 1988. Lac fusion analysis of the bet genes of *Escherichia coli*: regulation by osmolarity, temperature, oxygen, choline, and glycine betaine. J Bacteriol 170:5208–5215.

Falkenberg P, Strom AR. 1989. Purification and characterization of osmoregulatory protein betaine aldehyde dehydrogenase of *Escherichia coli*. Biochim Biophys Acta 1034:253–259.

Garcia-Perez A, Burg M. 1991. Renal medullary organic osmolytes. Physiol Rev 74:1081–1113.

Grossman EB, Hebert SC. 1989. Renal inner medullary choline dehydrogenase activity: characterization and modulation. Am J Physiol 256:F107–F112.

Hanson AD, Scott NA. 1980. Betaine synthesis from radioactive precursors in attached water-stressed barley leaves. Plant Physiol 66:342–348.

Hanson AD, Rhodes D. 1983. ^{14}C Tracer evidence for synthesis of choline and betaine via phosphoryl base intermediates in salinized sugarbeet leaves. Plant Physiol 71:692–700.

Hanson AD, May AM, Grumet R, Bode J, Jameison GC, Rhodes D. 1985. Betaine synthesis in chenopods: localization in chloroplasts. Proc Natl Acad Sci USA 82:3678–3682.

Haubrich DR, Gerber NH. 1981. Choline dehydrogenase: assay, properties and inhibitors. Biochem Pharmacol 30:2993–3000.

Hinkle DE, Wiersma W, Jurs S. 1998. Applied statistics for the behavioral sciences. 4th edition. New York: Houghton Mifflin Co.

Ikuta S, Imamura S, Misaki H, Horiuti Y. 1977. Purification and characterization of choline oxidase from *Artherobacter globiformis*. J Biochem 82:1741–1749.

Landfald B, Strom AR. 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J Bacteriol 165:849–855.

Mori N, Yoshida N, Kitamoto Y. 1992. Purification and properties of betaine aldehyde dehydrogenase from *Xanthomonas translucens*. J Ferment Bioeng 5:352–356.

Paynter KT. 1996. The effects of *Perkinsus marinus* infection on physiological processes in the eastern oyster, *Crassostrea virginica.* J Shellfish Res 15:119–125.

Paynter KT, Burreson EM. 1991. Effects of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*, II: disease development and impact on growth rate at different salinities. J Shellfish Res 10:425–431.

Paynter KT, Pierce SK, Burreson EM. 1995. Free amino acid levels in eastern oyster, *Crassostrea virginica* infected with *Perkinsus marinus*. Mar Biol 122:67–72.

Perrino LA, Pierce SK. 1999. Betaine aldehyde dehydrogenase kinetics partially account for oyster population differences in glycine betaine synthesis. J Exp Zool (in press).

Perroud B, LeRudulier D. 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J Bacteriol 161:393–401.

Petersen GL. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356.

Pierce SK. 1970. The water balance of *Modiolus* (Mollusca: Bivalvia: Mytilidae): osmotic concentrations in changing salinities. Comp Biochem Physiol 36:521–533.

Pierce SK, Rowland-Faux LM, O'Brien SM. 1992. Different salinity tolerance mechanisms in Atlantic and Chesapeake Bay conspecific oysters: glycine betaine and amino acid pool variations. Mar Biol 113:107–115.

Pierce SK, Rowland-Faux LM, Crombie BN. 1995. The mechanism of glycine betaine regulation in response to hyperosmotic stress in oyster mitochondria: a comparative study of Atlantic and Chesapeake Bay oysters. J Exp Zool 271:161–170.

Pierce SK, Dragolovich JD, Crombie BN. 1997. Variations in intracellular choline levels may account for the differences in glycine betaine synthesis rates between conspecific oyster populations responding to hyperosmotic stress. J Exp Zool 278:283–289.

Rowland LM, Pierce SK. 1985. The utilization and fate of quaternary ammonium compounds during low salinity adaptation in *Elysia chlorotica*. Physiol Zool 194:207–220.

Storey R, Wyn Jones RG. 1977. Quaternary ammonium compounds in plants in relation to salt resistance. Phytochemistry 16:447–453.

Stryer L. 1981. Biochemistry. 2nd edition. San Francisco: W.H. Freeman and Co.

Styrvold OB, Falkenberg P, Landfald B, Eshoo MW, Bjornsen T, Strom AR. 1986. Selection, mapping, and characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline-glycine betaine pathway. J Bacteriol 165:856–863.

Tombras-Smith L, Pocard JA, Bernard T, LeRudulier D. 1988. Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. J Bacteriol 170:3142–3149.

Weigel P, Weretilnyk EA, Hanson AD. 1986. Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiol 82:753–759.

Weretilnyk EA, Hanson AD. 1989. Betaine aldehyde dehydrogenase from spinach leaves: purification and in vitro translation of the mRNA, and regulation by salinity. Arch Biochem Biophys 271:56–63.

Weretilnyk EA, Smith DD, Wilch GA, Summers PS. 1995. Enzymes of choline synthesis in spinach. Plant Physiol 109:1085–1091.

Wood AJ, Saneoka H, Rhodes D, Joly RJ, Goldsbrough PB. 1996. Betaine aldehyde dehydrogenase in sorghum: molecular cloning and expression of two related genes. Plant Physiol 110:1301–1308.

Zhang J, Blusztajn JK, Zeisel SH. 1992. Measurement of the formation of betaine aldehyde and betaine in rat liver mitochondria by high pressure liquid chromatography-radioenzymatic assay. Biochim Biophys Acta 1117:333–339.