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Expression of Proteins Encoded by the *Escherichia coli cyn* Operon: Carbon Dioxide-Enhanced Degradation of Carbonic Anhydrase

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Cyanase catalyzes the reaction of cyanate with bicarbonate to give $2CO_2$. The *cynS* gene encoding cyanase, together with the *cynT* gene for carbonic anhydrase, is part of the *cyn* operon, the expression of which is induced in *Escherichia coli* by cyanate. The physiological role of carbonic anhydrase is to prevent depletion of cellular bicarbonate during cyanate decomposition due to loss of CO_2 (M. B. Guilloton, A. F. Lamblin, E. I. Kozliak, M. Gerami-Nejad, C. Tu, D. Silverman, P. M. Anderson, and J. A. Fuchs. J. Bacteriol. 175:1443–1451, 1993). A Δ*cynT* mutant strain was extremely sensitive to inhibition of growth by cyanate and did not catalyze decomposition of cyanate (even though an active cyanase was expressed) when grown at a low pCO_2 (in air) but had a Cyn^+ phenotype at a high pCO_2. Here the expression of these two enzymes in this unusual system for cyanate degradation was characterized in more detail. Both enzymes were found to be located in the cytosol and to be present at approximately equal levels in the presence of cyanate. A Δ*cynT* mutant strain could be complemented with high levels of expressed human carbonic anhydrase II; however, the mutant defect was not completely abolished, perhaps because the *E. coli* carbonic anhydrase is significantly less susceptible to inhibition by cyanate than mammalian carbonic anhydrases. The induced *E. coli* carbonic anhydrase appears to be particularly adapted to its function in cyanate degradation. Active cyanase remained in cells grown in the presence of either low or high pCO_2 after the inducer cyanate was depleted; in contrast, carbonic anhydrase protein was degraded very rapidly (minutes) at a high pCO_2 but much more slowly (hours) at a low pCO_2. A physiological significance of these observations is suggested by the observation that expression of carbonic anhydrase at a high pCO_2 decreased the growth rate.

Cyanase (EC 4.3.99.1) catalyzes the reaction of cyanate with bicarbonate to give $2CO_2$ (14) as follows: $NCO^- + 3H^+ + HCO_3^- \rightarrow 2CO_2 + NH_4^+$. This enzyme is induced in *Escherichia coli*, along with the enzyme carbonic anhydrase, by addition of cyanate to growth media (3, 12). The genes for these enzymes are part of the *cyn* operon, which includes three genes in the order *cynT*, *cynS*, and *cynX*, encoding carbonic anhydrase, cyanase, and a hydrophobic protein with an unknown function, respectively (12, 33–35). A recent study using chromosomal mutants constructed so that either the induced cyanase or carbonic anhydrase, respectively, was inactive provided evidence that the role of carbonic anhydrase is to prevent depletion of cellular bicarbonate during cyanate decomposition due to loss of CO_2, which diffuses out of the cells faster than noncatalyzed hydration back to bicarbonate. Thus, even though cyanase activity was induced in a Δ*cynT* mutant (nonfunctional carbonic anhydrase), the mutant strain was extremely sensitive to inhibition of growth by cyanate, did not catalyze decomposition of cyanate, and could not grow on cyanate as a sole source of nitrogen when grown under aeration with 0.03% CO_2 (in air). However, the $CynT^-$ phenotype was not present when aeration was done with 3% CO_2. Thus, the effect on the Δ*cynT* mutant strain of addition of cyanate to the culture medium is depletion of cellular bicarbonate, which leads to inhibition of growth and inability to catalyze cyanate degradation, and this effect can be overcome by addition of higher concentrations of CO_2 (13).

The studies reported here were carried out to characterize in more detail the expression of these two enzymes in this unusual bicarbonate-dependent system for cyanate degradation. Relative levels of expression and localization of the two enzymes in the matrix were established, complementation of the Δ*cynT* mutant with human carbonic anhydrase (demonstrating a property of the *E. coli* carbonic anhydrase unique to its role in cyanate metabolism) is described, and CO_2-dependent rapid degradation of carbonic anhydrase is reported.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used were derivatives of *Escherichia coli* K-12, as previously described (13). Construction of plasmid pCAH, which encodes a human carbonic anhydrase, is described below.

Media and chemicals. Glucose-minimal medium A supplemented with 4 μg of thiamine hydrochloride per ml (21) was used for physiological studies. Succinate (1 mM) was also added to all minimal growth media to permit growth when aeration was done with air containing 0% CO_2 (22). All of the other media, additions to culture media, and biochemicals and other supplies used were prepared or purchased as described previously (13). Strains growing in LB medium were tested for sensitivity to excess carbonic anhydrase by induction with 0.1 mM sodium azide, a gratuitous inducer (11). This concentra-

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tion of azide is sufficiently low that growth is not inhibited in a Cyn^+ strain but induction of the *cyn* operon occurs.

Growth conditions. For all growth studies, overnight cultures were used to inoculate 20 ml of medium in test tubes (25 by 200 mm) in a shaking water bath at 37°C. Mid-log-phase cultures were diluted in 20 ml of the same medium in test tubes (25 by 200 mm), placed in a 37°C water bath, and flushed continuously either with air (0.03% CO_2) or with air containing 3% CO_2. The optical density at 600 nm (OD_{600}) of each tube was monitored with a Spectronic SP-20 solid-state spectrophotometer. A linear relationship between cell density and OD was observed up to an OD of 0.8. To monitor the effect of cyanate on cell growth, KNCO was added to cultures when the OD_{600} was 0.1, and the OD was subsequently measured.

Detection of carbonic anhydrase and cyanase by Western blotting (immunoblotting). Antibodies were prepared and immunoblotting was carried out as previously described (13). Samples for blotting were prepared as follows. Liquid cultures (20 ml) were grown in minimal medium as described above. KNCO (0.5 mM) was added to cultures when the OD_{600} was 0.1. At appropriate times, cells were harvested, immediately suspended in 0.1 ml of denaturating buffer (0.05 M Tris [pH 6.8], 2.5% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol), and boiled for 1 min. Samples (5 to 10 μl) applied to the gel containing carbonic anhydrase (10 ng, unless indicated differently), cyanase (10 ng, unless indicated differently), or protein standards were also treated with denaturating buffer and boiled before application. For immunoblotting of carbonic anhydrase and cyanase in whole-cell extracts, some unexplained nonspecific binding was observed, but these interactions were with proteins that had mobilities different from that of either carbonic anhydrase or cyanase and did not interfere with interpretation of the results.

Samples of extracts of subcellular fractions were prepared in the same way. Cells suspended in 0.1 M potassium phosphate were disrupted by sonication in 0.1 M potassium phosphate buffer (pH 7.5). Low-speed centrifugation (to remove undisrupted cells) was carried out at 15,000 × *g* for 10 min; high-speed centrifugation (to sediment a membrane fraction) was carried out at 100,000 × *g* for 1 h. Pellets obtained after centrifugation were resuspended in the same volume as the original suspension (or the supernatant obtained after centrifugation).

Spheroplasts were prepared as described by Spizizen (30), with one minor modification—the spheroplasts were suspended in LB medium containing 0.1 mM sodium azide to facilitate continued induction of the *cyn* operon. Cells were harvested and then resuspended in LB medium containing 10 mM Tris-HCl and 7% sucrose, pH 8.0. Sodium azide, EDTA, and lysozyme were then added (at 0.1 mM, 10 mM, and 0.5 mg/ml, respectively). After incubation for 30 min at 26°C in a rotary shaker, spheroplasts were sedimented by centrifugation and the pellet was resuspended in the same buffer. Samples of the supernatant (periplasmic components) and suspended pellet (spheroplasts, representing cytosolic and membrane components) were subjected to Western blotting. Spheroplast formation was confirmed by centrifuging the suspension of spheroplasts, suspending the resulting pellet (spheroplasts) in water (which disrupts spheroplasts because of osmotic shock), centrifuging the suspension, and measuring the levels of enzymes in the supernatant and the pellet (resuspended in buffer and sonicated).

Purification of carbonic anhydrase and cyanase. Carbonic anhydrase was purified from strain SJ100/pAL4T as previously described (12). Cyanase was purified as previously described (33).

Assays of enzyme activities. Cyanase activity in vitro was determined as previously described (5, 6). Cells growing in a liquid culture were harvested and resuspended in 0.05 M phosphate buffer (pH 7.3), sonicated twice during 40 s on ice, and centrifuged for 10 min at 15,000 × *g*. A small, diluted volume of supernatant was assayed for cyanase activity. Cyanate decomposition in vivo was measured as described previously (13). Malate dehydrogenase activity was determined in 0.05 M phosphate buffer (pH 7.3) containing 0.15 mM NAD and 15 mM malate by measuring the rate of increase in A_{340}.

Complementation of Δ*cynT* mutant with human carbonic anhydrase. Plasmid pCAH, which encodes human carbonic anhydrase II under control of a T7 promoter, was transformed into Δ*cynT* mutant BUM012 (13) containing pGP1-2. pCAH was derived from a cDNA clone isolated by D. A. Jewell and P. J. Laipis, University of Florida; this bacterial expression vector is based on the pET system (32) and has been described by Tanhauser et al. (37). Plasmid pGP1-2 (36) encodes RNA polymerase under control of a thermolabile λ repressor. When grown at 30°C, BUM012 containing both plasmids had a $CynT^+$ phenotype whereas BUM012 with either plasmid alone had a $CynT^-$ phenotype.

RESULTS

Levels of expression and subcellular localization of induced cyanase and carbonic anhydrase. The relative concentrations of induced cyanase and carbonic anhydrase were estimated at the time of optimal expression of cyanase activity, which was 30 min after addition of cyanate to exponentially growing cells (at an OD of 0.1) in minimal medium. Extracts were prepared and subjected to Western blotting. The concentration of each enzyme in the extracts was estimated by comparison with known amounts of each enzyme run in parallel. As shown in Fig. 1, the levels of expression of the two enzymes were comparable, although the concentration of cyanase appeared to be about twice that of carbonic anhydrase. As noted in Fig. 1, both enzymes were present in the supernatant after sonication followed by low- and high-speed centrifugation (15,000 × *g* and 100,000 × *g*, respectively), suggesting that they are soluble proteins and are not tightly membrane associated.

Both enzymes were present in the spheroplasts harvested by centrifugation (an enzyme would have been in the supernatant if it had been localized in the periplasmic space). After dilution of the spheroplasts in cold water (resulting in osmotic disruption of the spheroplasts [30]), followed by centrifugation, both enzymes were found only in the supernatant. The presence of cyanase and carbonic anhydrase in the different fractions was established by assay (cyanase only) and Western blotting (data not shown). The localization of both enzymes correlated with the localization of malate dehydrogenase activity, which is localized in the matrix as a tricarboxylic acid cycle enzyme (23).

Complementation of a Δ*cynT* mutant strain (BUM012) with a plasmid harboring human carbonic anhydrase. As reported previously and as shown in Fig. 2, growth of mutant strain BUM012 (Δ*cynT*) is extremely sensitive to cyanate compared with that of the parental wild-type (WT) strain (BUM015) and cyanate is not broken down even though cyanase is expressed; since cyanate is not degraded, this strain cannot, therefore, grow on cyanate as the sole source of nitrogen, as can the parental strain (13). However, transformation of this strain with a plasmid expressing human carbonic anhydrase II results in a strain in which growth is less sensitive to cyanate inhibition and which degrades cyanate (Fig. 2). As expected, the transformed strain also grew on cyanate as the sole source of nitrogen (data not shown). Thus, the cyanate-induced *E. coli*

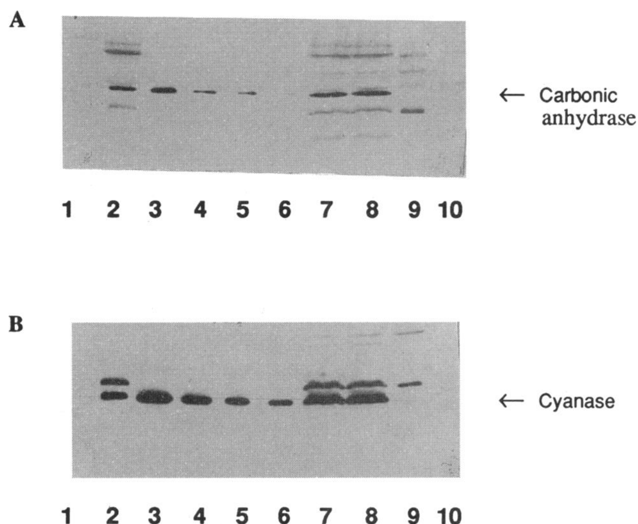

FIG. 1. Immunoblots of purified carbonic anhydrase and cyanase and of induced whole-cell extracts before and after centrifugation. WT (BUM015) cells were grown in glucose-minimal medium (20 ml) with shaking and induced with 0.5 mM cyanate when the OD_{600} was 0.1. Samples were prepared as described in Materials and Methods. Dilutions of suspended pellets were adjusted so that the samples of either pellets or supernatant added to the gels represented the same volumes of the original culture. (A) Carbonic anhydrase immunoblots. Lanes: 1, standard protein molecular weight markers (locations are not indicated); 2, WT (BUM015) whole-cell extract; 3 to 6, 7.6, 3.8, 1.9, and 0.76 ng of purified carbonic anhydrase, respectively; 7, supernatant after centrifugation at 15,000 × *g*; 8, supernatant after centrifugation at 100,000 × *g*; 9, suspended pellet after centrifugation at 15,000 × *g*; 10, suspended pellet obtained after centrifugation at 100,000 × *g*. (B) Cyanase immunoblots. Lanes 1 to 10 were as described for panel A, except for lanes 3 to 6, which contained 40, 20, 10, and 4 ng of cyanase, respectively.

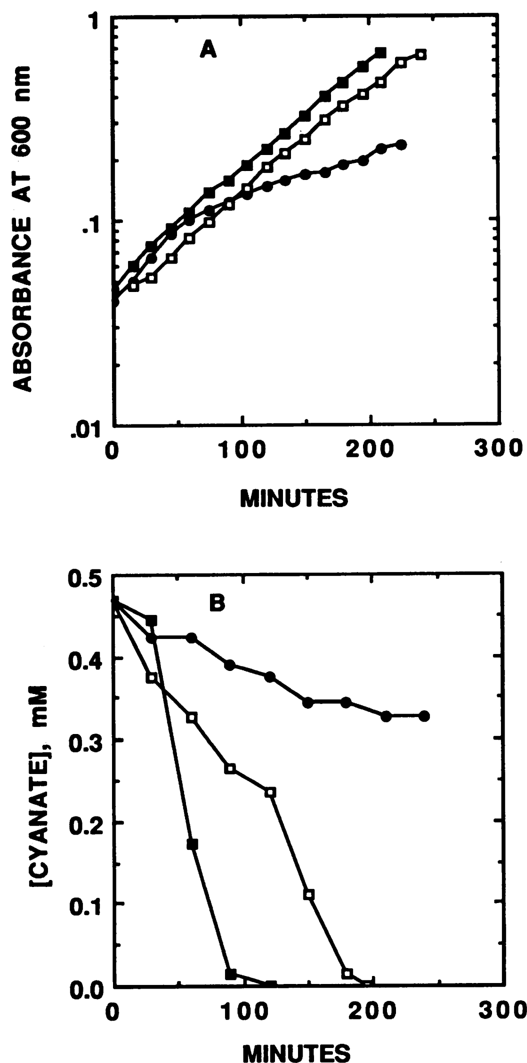

FIG. 2. Effect of complementation of BUM012 (Δ*cynT*) by transformation of BUM012 with pCAH. Growth in glucose-minimal medium was done as described in Materials and Methods. Cyanate (0.5 mM) was added when the OD_{600} was 0.1. (A) Effect of cyanate on growth. (B) Cyanate decomposition; time zero represents the time of cyanate addition. Symbols: ■, parental WT strain; ●, BUM012 (Δ*cynT*); □, BUM012/pCAH/pGP1-2.

carbonic anhydrase is not uniquely essential for normal function. However, the results in Fig. 2 suggest that the *E. coli* enzyme is particularly adapted to its function in cyanate degradation. Induced *E. coli* carbonic anhydrase cannot be detected in the parental WT strain by using routine titration methods; this method can detect induced *E. coli* carbonic anhydrase only in strains such as SJ100/pAL4T, in which the carbonic anhydrase is encoded by a plasmid and is highly overexpressed, as we have demonstrated previously (12). Calculation of carbonic anhydrase activity on the basis of the level of carbonic anhydrase protein from the Western blotting experiments described in Fig. 1 and the specific activity of the purified enzyme indicated that the total carbonic anhydrase activity from 20 ml of a culture of the induced WT strain was about 1 order of magnitude lower than that needed for detection by this assay (12). In contrast, carbonic anhydrase activity from the same culture volume (20 ml) at the same level of growth was easily detectable for strain BUM012/pCAH/pGP1-2. The calculated carbonic anhydrase activity in the latter case was more than 50 times that present in the original parental strain (BUM015), yet cyanate decomposition was lower than observed for BUM015 (Fig. 2). Thus, the calculated activity of human carbonic anhydrase in the transformed mutant strain was considerably higher than that induced in BUM015 yet the mutant defect was not completely abolished.

Dependence of rate of carbonic anhydrase and cyanase degradation on pCO_2. Western blotting experiments with whole-cell extracts showed that after the disappearance of the inducer cyanate, carbonic anhydrase was degraded at a much faster rate than cyanase and the rate of degradation was dependent upon the pCO_2. In cultures aerated with 3% CO_2, carbonic anhydrase was not detectable 30 min after cyanate had disappeared, whereas the level of cyanase remained essentially constant for at least 180 min after the disappearance of cyanate (Fig. 3C and 4B, respectively). Cyanase degradation did not appear to be affected by the pCO_2 (Fig. 4). As shown in Fig. 3 and 4, however, the rapid disappearance of carbonic anhydrase appears to be dependent on the pCO_2. In cultures aerated with 0.03 and 0% CO_2 (Fig. 3B and A, respectively), the presence of significant levels of carbonic anhydrase were detected in extracts at progressively later times after cyanate had disappeared. In these studies, the time required to degrade added cyanate became longer as the pCO_2 decreased (about 25, 75, and 95 min at 3, 0.03, and 0% CO_2, respectively). The

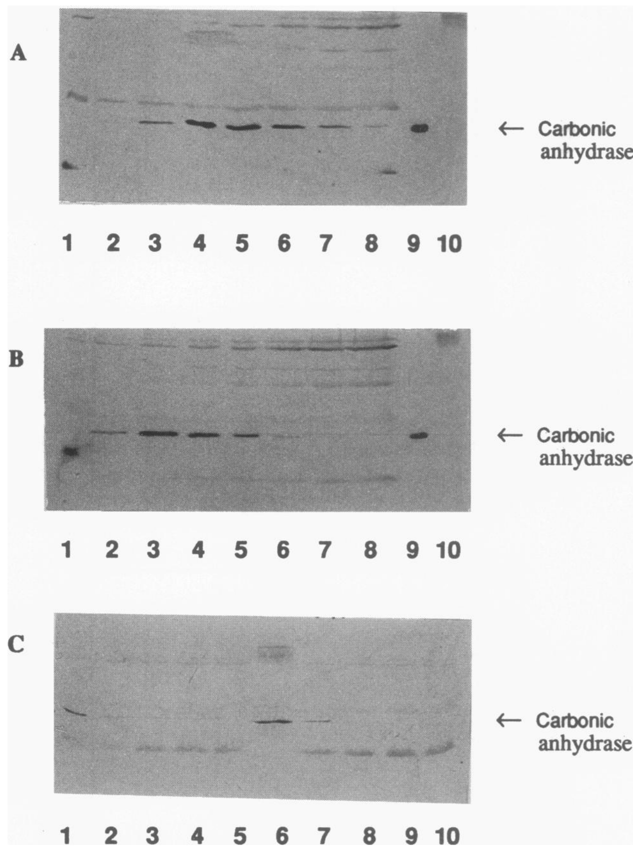

FIG. 3. Immunoblots of purified carbonic anhydrase and of whole-cell extracts at various times after induction during aeration with 0, 0.03, or 3% CO_2. Unless indicated otherwise, all of the cultures were with parental WT strain BUM015. All of the cultures were grown in glucose-minimal media and induced by 0.5 mM cyanate when the OD_{600} was 0.1. Aliquots (10 ml) of the liquid culture were removed at 30-min intervals after induction and immediately harvested by centrifugation, and the pellet was extracted and subjected to immunoblotting as described in Materials and Methods. (A) 0% CO_2. Lanes: 1 to 8, whole-cell extracts at 0, 30, 60, 90, 120, 150, 180, and 240 min after induction, respectively; 9, purified carbonic anhydrase; 10, standard protein molecular weight markers (locations are not indicated). (B) 0.03% CO_2. Lanes: 1 to 8, whole-cell extracts at 0, 30, 60, 90, 120, 150, 180, and 210 min after induction, respectively; 9 to 10, same as for panel A. (C) 3% CO_2. Lanes: 1 to 5, whole-cell extracts of BUM019 (*cynX::kan*) at 30, 60, 90, 120, and 150 min; 6, purified carbonic anhydrase; 7 to 10, whole-cell extracts of BUM015 (WT) at 30, 60, 90, and 120 min after induction, respectively.

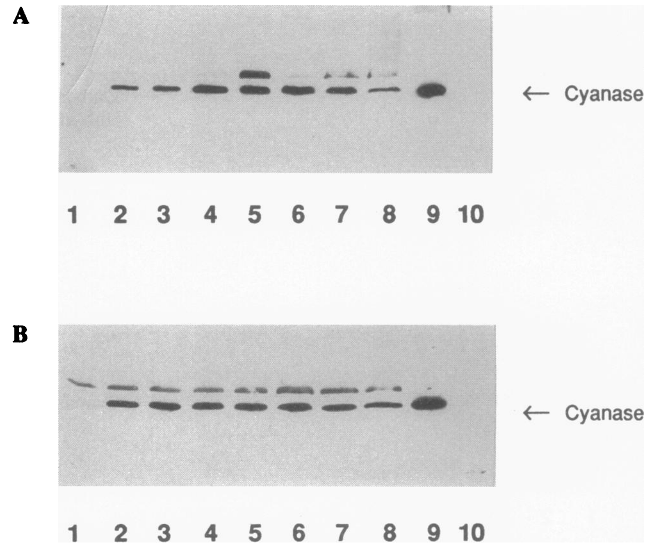

FIG. 4. Immunoblots of purified cyanase and of whole-cell extracts at various times after induction during aeration with 0.03 or 3% CO_2. Samples were prepared as described in the legend to Fig. 3. (A) 0.03% CO_2. Lanes: 1 to 8, whole-cell extracts at 0, 30, 60, 90, 120, 150, 180, and 210 min after induction, respectively; 9, purified cyanase; 10, standard protein molecular weight markers (locations are not indicated). (B) 3% CO_2. Lanes 1 to 10 were the same as in panel A.

consequent lag in growth was also longer, presumably because of a corresponding decrease in the concentration of cellular bicarbonate. Nevertheless, the growth rates of the cultures were comparable after cyanate had been degraded. It is clear that carbonic anhydrase persists for a significantly longer period of time at a low pCO_2 than at a high pCO_2 after cyanate has been degraded.

These studies assumed that the effects of pCO_2 are on degradation of carbonic anhydrase after the inducer cyanate is depleted, i.e., induction of the *cyn* operon ceases and existing *cyn* operon transcripts turn over rapidly. This was confirmed by experiments in which chloramphenicol (at 100 μg/ml, which stopped further protein synthesis) was added just before cyanate was completely degraded (however, degradation of cyanate continued and was completed in the same time period because of the presence of endogenous cyanase at the time of addition of chloramphenicol). Results virtually identical to those shown in Fig. 3 and 4 were obtained.

The results in Fig. 3 and 4 show measured cyanase and carbonic anydrase proteins, not activity. When cyanase activity was measured, the results correlated with the stability of the cyanase protein after all of the cyanate had been degraded, i.e., total cyanase activity did not decline after cyanate was degraded. This is consistent with our experience in isolating active cyanase from large volumes of induced cells harvested several hours after cyanate had been degraded (3). Of course, disappearance of carbonic anhydrase protein (Fig. 3 and 4) would be accompanied by a corresponding decrease in carbonic anhydrase activity. As noted above, the level of carbonic anhydrase activity induced in WT *E. coli* was too low to be measured by standard methods. However, retention of carbonic anhydrase activity along with the observed retention of carbonic anhydrase protein in WT cells grown with no CO_2 after new protein synthesis was inhibited by addition of chloramphenicol was demonstrated indirectly as follows. WT cells grown with no CO_2 were induced by addition of cyanate as described in Fig. 3A for 30 min, at which time the cyanase and carbonic anhydrase proteins were present but little cyanate had been degraded (note the lag period described above). Chloramphenicol was then added to stop further protein synthesis. Cyanate degradation continued (although more slowly than observed in Fig. 3A because of the lower levels of both carbonic anhydrase and cyanase induced under these conditions) and was complete after 4 h. If carbonic anhydrase activity had disappeared, cyanate degradation would have stopped; this situation would be analogous to the inability of the Δ*cynT* mutant to degrade cyanate at a low pCO_2 (13). This expectation was confirmed by control experiments in which the Δ*cynT* mutant strain was used instead of WT cells under the same conditions and by growing WT cells at a high pCO_2 and then transferring them to a medium containing chloramphen-

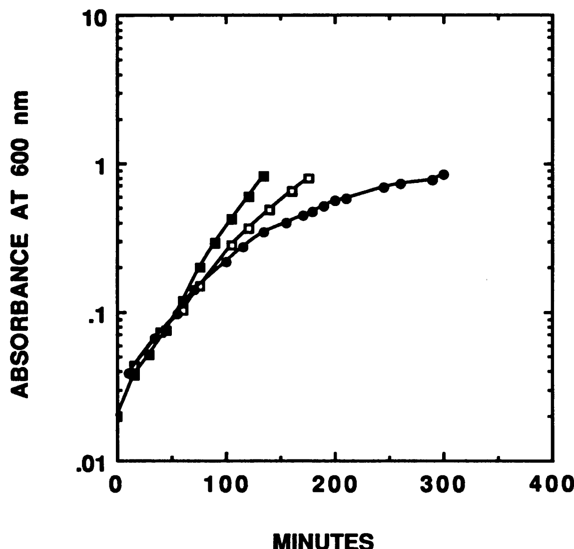

FIG. 5. Effect of pCO_2 on the growth of strain SJ100/pAL4T in the presence or absence of azide, a gratuitous inducer of the carbonic anhydrase encoded by pAL4T. Growth in LB medium was done as described in Materials and Methods; azide (0.1 mM) was added when the OD_{600} was 0.1. Symbols: ■, 0.03% CO_2; □, 3% CO_2; ●, 3% CO_2 with induction by azide.

icol and cyanate at a low pCO_2 shortly after all of the carbonic anhydrase protein had been degraded; in both cases, there was very little subsequent cyanate degradation. Although these indirect experiments do not provide information about the relationship between carbonic anhydrase activity and carbonic anhydrase protein in the absence of cyanate, they do indicate that carbonic anhydrase protein that remains in cells for an extended period of time when cells are grown with no CO_2 after further carbonic anhydrase synthesis is stopped retains activity.

The results in Fig. 3C indicate that the rapid degradation of carbonic anhydrase at a high pCO_2 also occurred in mutant strain BUM019 (*cynX*::*kan*), in which the product of the *cynX* gene is nonfunctional (13).

As shown in Fig. 5, a high pCO_2 (3%) alone had a small inhibitory effect on the growth of strain SJ100/pAL4T. This inhibitory effect was significantly enhanced by azide induction of the plasmid-borne carbonic anhydrase. Azide had no effect on the growth of SJ100/pAL4T at 0.03% CO_2. A high pCO_2 (3%) also had a similar, small inhibitory effect on the growth of SJ100 (Δ*cyn*), as well as on that of the other strains tested (data not shown). Azide at the concentration used in these studies had no effect on the growth of SJ100 (Δ*cyn*) at either a high pCO_2 (3%) or a low pCO_2 (0.03%) (data not shown). Thus, azide at this concentration is not toxic for cell growth at either a high or a low pCO_2 and excess carbonic anhydrase does not affect growth at a low pCO_2. Carbonic anhydrase (expressed at higher levels in SJ100/pAL4T than in WT strain BUM015) apparently becomes inhibitory to growth only when the pCO_2 in the aerating gases is high. This same effect (i.e., inhibition of growth at a high pCO_2 after excess carbonic anhydrase activity has been induced in LB medium) has also been observed in a Δ*cyn* strain complemented with human carbonic anhydrase (unpublished observation). We have observed these effects only in enriched medium.

DISCUSSION

The task of degrading cyanate appears to invoke induction of approximately equal levels of carbonic anhydrase and cyanase. The catalytic turnover value per subunit of carbonic anhydrase is approximately 50 times the turnover value per subunit of cyanase determined under our defined assay conditions in vitro (12), but this value is probably lower in cells, depending on the degree of saturation of CO_2 in the cells and inhibition of carbonic anhydrase by cyanate. Additional considerations include (i) the fact that one turnover of cyanase results in formation of two equivalents of CO_2 and (ii) the rate of degradation of carbonic anhydrase even when cyanate is still present may be somewhat faster than that of cyanase so that the ratio of the concentration of each, respectively, is lower than 1 (the latter point may explain the somewhat lower level of carbonic anhydrase than cyanase at the time of optimal expression [Fig. 1]). Consequently, while significant levels of cyanate are still present and cyanate is being degraded by cyanase, it appears that the system for cyanate degradation maintains a level of carbonic anhydrase perhaps just sufficient to ensure that bicarbonate levels are not depleted, which would prevent cyanate degradation.

The carbonic anhydrase from *E. coli* has significant sequence identity to plant carbonic anhydrases, which are quite different from mammalian carbonic anhydrases (12, 19, 38). One feature of the *E. coli* carbonic anhydrase that is uniquely applicable to its role in recycling of CO_2 during cyanate degradation is its higher anion inhibition constant for cyanate inhibition than mammalian carbonic anhydrases (12, 20). Clearly, this would be an important feature of *E. coli* carbonic anhydrase, which is called upon to function only when cyanate is present. The complementation study reported here is consistent with this interpretation. Human carbonic anhydrase can substitute for the *E. coli* enzyme, permitting growth and cyanate decomposition when cyanate is added to cells. However, the rate of cyanate decomposition is not as high as with the original parental WT strain, even though the expressed activity is considerably higher than in the induced WT strain. It is likely that this reflects the marked inhibition of human carbonic anhydrase by the cyanate present in the cells.

Complementation of the Δ*cynT* mutant with a human carbonic anhydrase as described here suggests that this system could be a valuable tool for screening for carbonic anhydrase-encoding genes in cDNA libraries, as well as for identification of mutated cloned genes. This would be particularly useful for the many systems in which detection of carbonic anhydrase activity is difficult with conventional assay methods.

The observation that carbonic anhydrase is markedly more susceptible than cyanase to in vivo proteolytic degradation may have physiological significance specifically relevant to this system. One explanation for a need for rapid proteolytic degradation of carbonic anhydrase is the possibility that high levels of carbonic anhydrase may enhance the known inhibitory effects of a high pCO_2 on growth (8, 16), as shown in Fig. 5. The fact that the rate of degradation increases as the external pCO_2 increases fits the logic of this suggestion. With high levels of both cyanase and carbonic anhydrase activities present, the level of bicarbonate will increase, perhaps beyond what is useful for the cell, thus signaling the degradation of carbonic anhydrase and consequently allowing some of the bicarbonate (CO_2) derived from cyanate to escape. It has been reported previously that bicarbonate exhibits significant competitive substrate inhibition of cyanase, particularly if the levels of cyanate are low (5). Thus, the dependence of carbonic anhydrase degradation on the bicarbonate-CO_2 concentration

may allow maintenance of a balance of bicarbonate that is not too low or too high, thus preventing inhibition of growth and maximizing the rate of cyanate decomposition (for different reasons in each case). By contrast, the long-term stability of cyanase may also be beneficial, allowing continued degradation of low levels of cyanate under conditions (low rate of degradation or high pCO_2) in which recycling of CO_2 is not necessary.

The molecular mechanism(s) that signals carbonic anhydrase degradation is not known. The state of association of carbonic anhydrase is markedly affected by the bicarbonate-CO_2 concentration. The enzyme exists as a tetramer in the absence of added bicarbonate but as a dimer in the presence of added bicarbonate (5 mM) (12); perhaps the latter state of association is more susceptible to proteolysis. Under conditions of rapid cyanate decomposition catalyzed by cyanase in the presence of carbonic anhydrase, the resulting higher levels of bicarbonate-CO_2 in the cell should tend to signal degradation of carbonic anhydrase, as suggested above. The levels of cyanate may also play a role in regulating the rate of carbonic anhydrase degradation. Reversible inhibition of the thiol protease papain by a low concentration (0.1 mM) of cyanate has been reported (29). A possible consideration consistent with the above views is that carboxylation of amino or other functional nucleophilic groups of key proteases by higher concentrations of CO_2 may occur, resulting in conformational changes that affect activity. Such carboxylation reactions with physiological consequences have been reported (17, 25, 27). Cyanate also reacts with these same functional nucleophilic groups (4, 29, 31) and cyanate may, therefore, compete with such carboxylation reactions. The possibility that the product of *cynX* (protein X) (35) is a protease specific for carbonic anhydrase subject to activation by a high pCO_2 was investigated by comparing the rates of carbonic anhydrase degradation in WT and *cynX::kan* mutant strains (Fig. 3C). However, no difference in the rates of disappearance of carbonic anhydrase at a high pCO_2 was detected, which is consistent with previous results suggesting that the product of gene X does not appear to affect cyanate metabolism (13).

The conclusion reached here, that both induced enzymes are localized in the cytosol, is consistent with previously reported data showing biphasic kinetics for depletion of ^{18}O from CO_2 in suspensions of cells (13). Depletion of ^{18}O from CO_2 in suspensions of cells containing intracellular carbonic anhydrase (with no carbonic anhydrase in the extracellular fluid) is characterized by biphasic kinetics; that is, the decreasing atom fraction of ^{18}O in CO_2 is described by the sum of two exponentials (9, 28). This arises because CO_2 is readily accessible to the intracellular enzymes but HCO_3^- is not. The observation of biphasic ^{18}O loss from CO_2 in suspensions of *E. coli* with induced carbonic anhydrase (13) indicates the compartmentalization of this enzyme in a semipermeable space, suggesting that it is localized in the cytosol and not in the periplasmic space. The localization of both carbonic anhydrase and cyanase in the cytosol, apparently in soluble form, is not unexpected and is physiologically consistent. This ensures an available supply of the bicarbonate needed for cyanate decomposition. Secondly, it allows the apparent regulation of carbonic anhydrase activity by adjustment of the rate of its degradation as described above.

On the other hand, the localization of induced *E. coli* carbonic anhydrase in the cytosol may stand in contrast to that of the few other carbonic anhydrases which have been characterized in prokaryotes, in which carbonic anhydrases are apparently not localized in the cytosol (1, 2, 10, 15, 18, 26). In this context, we call attention to the structural properties of the

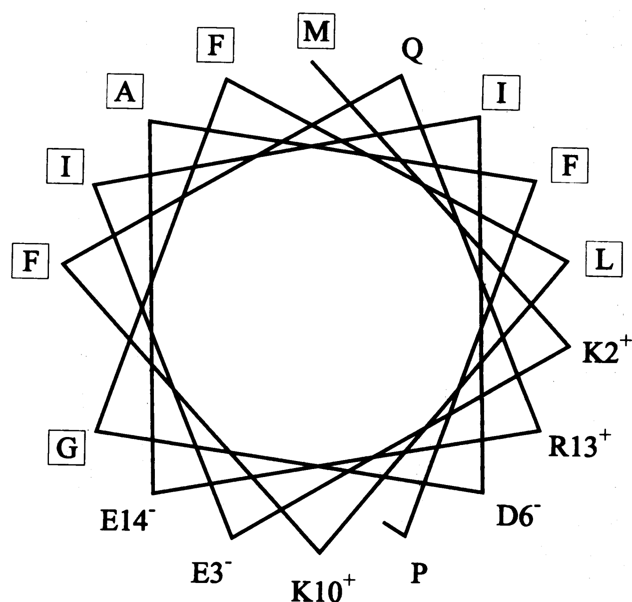

FIG. 6. Helical-wheel representation of the 17-amino-acid N-terminal sequence of *E. coli* carbonic anhydrase as an α-helix. Hydrophobic amino acid residues are boxed. Charge polarity and position with respect to the N-terminal end are indicated for the hydrophilic amino acid residues.

N-terminal sequence of *E. coli* carbonic anhydrase, which poses possibly interesting questions concerning the function and evolution of this operon. Chou-Fasman analysis of secondary structure predicts that the 17-amino-acid N-terminal sequence has a high propensity to form an α-helix (data not shown). As illustrated in Fig. 6 in a helical-wheel format of an α-helix, this segment is composed almost entirely of hydrophobic residues and the other side is composed only of charged or polar residues. This could simply reflect the localization of this N-terminal sequence on the surface of the protein as an α-helix. Alternatively, a parallel association of several of these helix segments could give a structure with a charged hydrophilic interior and a hydrophobic exterior that might associate with a cell membrane. Our observations that the isolated carbonic anhydrase associates to tetramer when the bicarbonate-CO_2 concentrations are low (4) and that even at low concentrations the enzyme tends to aggregate and precipitate under these conditions (unpublished observations) are consistent with this kind of association. The charged residues on the hydrophilic side of the helix segment are arranged symmetrically on each side of an axis perpendicular to the helix and passing from the center of the helix through residue 17, proline ($K2^+$, $R13^+$, $D6^-$, proline, $K10^+$, $E3^-$, $E14^-$). This could reflect a specific arrangement for stabilization of the oligomer through ionic interactions between subunits ($K2^+$ to $E3^-$ and $R13^+$ to $E14^-$) with a cluster of negative charges ($[D6^-]_n$ [n is the number of subunits]) and positive charges ($[K10^+]_n$ in the center. Perhaps this N-terminal sequence represents a vestigial peptide that facilitated specific membrane association of the carbonic anhydrase (perhaps with assistance from the product of *cynX*, which now appears to serve no purpose) but lost this function during evolution. *E. coli* cells may have needed this localization of carbonic anhydrase to facilitate, in conjunction with the activity of carbonic anhydrase, transport of bicarbonate into cells for cyanate decomposition. Association of carbonic anhydrase with bacterial and mammalian cell mem-

branes has been reported (7, 15, 18, 26); these associations are thought to be essential for membrane transport of bicarbonate to optimize the cellular level of bicarbonate-CO_2. A membrane-bound, as well as a soluble, carbonic anhydrase functions in cyanobacteria to transport bicarbonate into cells, where it is converted to CO_2 and concentrated in the carboxysome for ribulose bisphosphate carboxylase activity (10, 26).

In the context of the above discussion, the possibility also remains that *E. coli* carbonic anhydrase is loosely bound to the inner membrane, perhaps under specific circumstances, e.g., when bicarbonate-CO_2 levels are low. Our initial efforts to establish the subcellular localization of carbonic anhydrase and cyanase utilized an osmotic shock approach (24). A significant difference between the distribution of cyanase (distributed between the pellet and supernatant after centrifugation) and that of carbonic anhydrase (found predominantly in the supernatant) was, in fact, consistently observed (unpublished results), suggesting that the carbonic anhydrase was more closely associated with the membrane, thus facilitating release. However, it has been pointed out that ambiguities are common when this approach is used (24).

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