Osmotic stress induces variation in cellular levels of ornithine decarboxylase-antizyme

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The polyamines, and especially putrescine, play an integral role in the physiological response of cells to varying extracellular osmotic conditions. Ornithine decarboxylase (ODC) synthesis and stability, as well as the activity of the polyamine transporter, had all been reported to be very sensitive to media osmolarity in different cells and tissues, yet the mechanism of this complex, co-ordinated response was not known. In this study we have determined that all these aspects of osmotic-shock response may be mediated by the common regulatory protein, ODC-antizyme. HTC cells were induced for antizyme and then exposed to media of reduced osmotic strength. Both antizyme activity and protein decreased rapidly, under these conditions, to new steady-state levels that depended upon the degree of reduction in media tonicity. This antizyme reduction was found to be due to a rapid increase in antizyme degradation, with a half-life decrease from 75 min down to 45 min occurring immediately upon exchanging media. In complementary experiments, increased media tonicity induced elevated antizyme levels and stability. The sensitivity of antizyme turnover to osmotic conditions was also observed in DH23b cells, which contain elevated levels of more stable antizyme. Interestingly, the two main antizyme proteins, AZ-1 and AZ-2 (presumably products from the first and second translational start sites), differed in their responses to these changing osmotic conditions. Just as feedback regulation of antizyme synthesis provides an effective mechanism for maintaining stable polyamine levels, these studies suggest that alteration in the rate of antizyme degradation may be the mechanism whereby cells adjust steady-state polyamine levels in response to stimulation or stress.

INTRODUCTION

Mammalian cells subjected to acute hypo-osmotic stress respond with rapid inorganic ion fluxes, a net loss in the K^+ ion, and increased synthesis and uptake of the polyamine putrescine. The polyamines, and especially putrescine, are multivalent organic cations that serve a necessary physiological function in the accommodation of both bacterial [1] and mammalian [2–6] cells to the stress of varying environmental osmotic conditions. In the last 30 years there have been many studies concerning changes in polyamine metabolism associated with osmotic stress, yet the mechanism of this response is still poorly understood.

The enzyme responsible for mammalian putrescine synthesis, ornithine decarboxylase (ODC; E. C. 4.1.1.17), increases sharply in hypotonic culture conditions and decreases in hypertonic media [2–4,7–10]. Though Lundgren [11] suggests that this control is exerted at the level of transcription, most investigators find that the response of ODC to varying osmotic conditions is post-transcriptional, noting major changes in both translation of the ODC message and stability of the enzyme [2,4,8–10]. In addition to these changes in polyamine biosynthesis, cells also respond to osmotic stress by rapidly varying polyamine transport. Although putrescine levels are probably most crucial to the accommodation of cells to osmotic changes, the uptake of all the polyamines seem to be increased in hypo-osmotic conditions [1,3,5,6,8,12], as they appear to share a common transport system.

At the outset it would appear that three different mechanisms would be required to explain the effects of varying osmotic strength on the biochemically distinct processes of ODC translation, ODC degradation and polyamine transport. Recent evidence, however, suggests the possibility that these three processes may all be linked by a common regulatory intermediate. It has been known for some time that the rapid degradation of ODC is controlled by the level of a small regulatory protein, ODC-antizyme, whose synthesis is tightly directed by intracellular concentrations of free spermidine (reviewed in [13,14]). Other studies have shown that this same antizyme is responsible for apparent variations in the rate of ODC-mRNA translation [15,16]. Most recently, a rather unexpected connection has been discovered between antizyme and polyamine transport. Although the mammalian cytoplasmic membrane polyamine transporter has not yet been isolated, experiments using cells transfected with an inducible antizyme gene indicate that antizyme is involved in the rapid, reversible modulation of the activity of this transporter [17–19]. These studies suggest that antizyme could very well be the common link allowing co-ordination between these various aspects of polyamine level control and osmotic stress. Against this hypothesis is the fact that the only known regulator of antizyme activity is the level of free cellular spermidine, and this does not appear to change during osmotic stress [1,4,8,9].

We have now tested this hypothesis and found that antizyme levels do indeed vary in relation to the osmotic strength of a cell's medium. Further, we have discovered that this change is the result of variation in the stability of this antizyme, an entirely new mode of control of this very critical regulatory protein.

EXPERIMENTAL

Cell culture

Rat hepatomal (HTC) cells were grown in monolayer and suspension cultures in Swim's 77 medium containing 10% (v/v) calf serum as previously described [18]. ODC-stable and ODC-overproducing DH23b cells were selected from HTC cells as described previously [20], and cultures were maintained as for the parental line, except for the presence of 10 mM difluoromethylornithine (Eflornithine; kindly provided by the Marion Merrell

Abbreviations used: ODC, ornithine decarboxylase.
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Dow Research Institute, Cincinnati, OH, U.S.A.) in the medium. Hypo-osmotic culture conditions were generated by indicated dilutions of the Swim's 77 medium with sterile, deionized water containing an equivalent concentration of the antizyme-inducing polyamine. Where indicated the medium was replaced with pre-warmed, sterile, PBS.

Antizyme assay

Cell pellets were homogenized by brief sonication in 0.02 M potassium phosphate buffer (pH 7.0) containing 2.0 mM dithiothreitol and 0.2 mM EDTA. Samples of the cell homogenate were mixed with 0.5 unit of ODC produced in HTC cells in 0.1 ml of 0.02 M Mops buffer (pH 7.2) containing 0.5 mM EDTA, 1.0 mM dithiothreitol and 0.02 % Triton X-100. After 15 min on ice, additional Mops buffer was added to 0.175 ml total volume, and the mixture was assayed for ODC activity as described previously [21]. In essence, the assay measures the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine. One unit of ODC is defined as the amount of enzyme required to release of 1 nmol of $^{14}CO_2$/h. Antizyme activity was determined by measuring the loss of ODC activity attributed to antizyme addition. As indicated previously [21], inhibition of ODC activity up to 75 % was linear with respect to the amount of antizyme added. One unit of antizyme activity is defined as the amount required to inhibit one unit of ODC.

Immunodetection of antizyme protein

Affinity-purified polyclonal rabbit antibody specific for antizyme was prepared by using an antizyme fusion protein expressed in bacteria as previously described [18]. Frozen cell pellets were suspended in SDS sample buffer, and aliquots containing about 80 μg of protein (15 μg for DH23b cell samples) were applied to SDS/12 % polyacrylamide gels. Protein bands were transferred to a nitrocellulose filter (Hoefer), stained with Ponceau S to reveal the molecular-mass standards and blocked with 5 % non-fat dried milk. The blots were exposed to affinity-purified antibody against antizyme overnight at room temperature. Bands were revealed by the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad). In order to evaluate the relative band intensities, the blots were scanned using Photoshop 3.0 and an Envisions 8100 scanner. The images were then analysed using Image Calc by C. H. A. van de Lest [21a].

RESULTS

Detection of altered antizyme levels in HTC cells subjected to a hypo-osmotic shift in medium

Both ODC and its regulatory protein, antizyme, are present in very low concentrations in mammalian cells and, even when induced, constitute only about 3–5 ng/mg of cell protein. Detection of antizyme is especially difficult, and its activity can only be bioassayed by the ability of free molecules of antizyme to bind and inactivate ODC. Not only is antizyme protein present in very low levels, but it is also separated into two different translation products [22,23], and additional unidentified minor bands are visible using SDS/PAGE [24]. To facilitate assay and immunodetection of this scarce protein, these experiments were conducted on cells in which antizyme had been induced by addition of either putrescine or spermidine. Though these conditions allow evaluation of the control of antizyme levels, they preclude observation of any changes in ODC translation and stability, or polyamine-transport activity that can be detected under more reduced antizyme levels. It is reasonable to assume that factors that influence antizyme synthesis and stability under conditions where it is highly induced would be equally effective in controlling the reduced antizyme levels observed in the presence of active ODC.

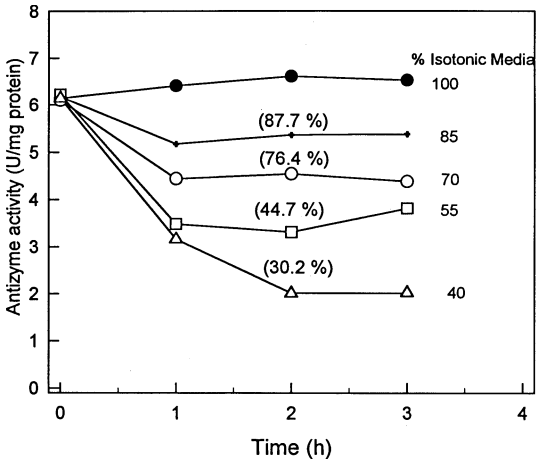

Figure 1 Decrease in antizyme following transition to hypo-osmotic culture medium

Antizyme was induced in HTC cell cultures by the addition of 5 mM putrescine. After 18 h the medium was removed, diluted to the indicated levels (noted as a percentage of the isotonic medium) with sterile deionized water containing 5 mM putrescine, and replaced. Samples taken at hourly intervals were assayed for antizyme activity. Duplicate samples taken at 2 h of hypo-osmotic treatment were evaluated for relative antizyme protein levels as determined by immunodetection of the 24 kDa antizyme band on Western blots. The relative antizyme band intensities at 2 h are indicated in parentheses as percentages of the undiluted control. U, units.

Rat hepatoma HTC cells were induced for antizyme by the addition of 5 mM putrescine and subsequently were exposed to various hypo-osmotic media. This change in osmotic strength was accomplished by diluting normosmotic medium with increasing amounts of deionized water that also contained the antizyme-inducing 5 mM putrescine. Such medium dilution has been used previously in experiments demonstrating hypo-osmotic stress responses [3,7,25]. As seen in Figure 1, these conditions induced a rapid and graded decrease in antizyme activity, establishing a new, lowered, steady-state level of antizyme, even though the cells were still viable (as indicated by dye exclusion) and continuously exposed to exogenous putrescine. Further, this decrease in antizyme activity was accompanied by an equivalent depressed level of antizyme protein, as determined by immunodetection of samples removed 2 h after the osmotic shift. For example, under moderate hypotonic shock where the osmotic strength was reduced to about 55 % of normosmotic conditions, there was greater than a 50 % loss in antizyme activity and an approx. 55 % decrease in antizyme protein. Since antizyme is considered to act catalytically in stimulating ODC-protein degradation, and perhaps also in inhibiting ODC-mRNA translation, this magnitude of decrease in antizyme could easily explain the increases in ODC activities reported in past studies.

Mechanism of the rapid decrease in antizyme in response to hypo-osmotic shock

A reduction to 60 % normosmotic strength was used in the subsequent osmotic shock experiments, as this routinely reduced antizyme levels by about 50 % within 1 h of treatment (Figure 2A). To assess whether this loss in antizyme reflected decreased synthesis or increased degradation, replicate cultures were subjected to this osmotic stress at the same time that cycloheximide

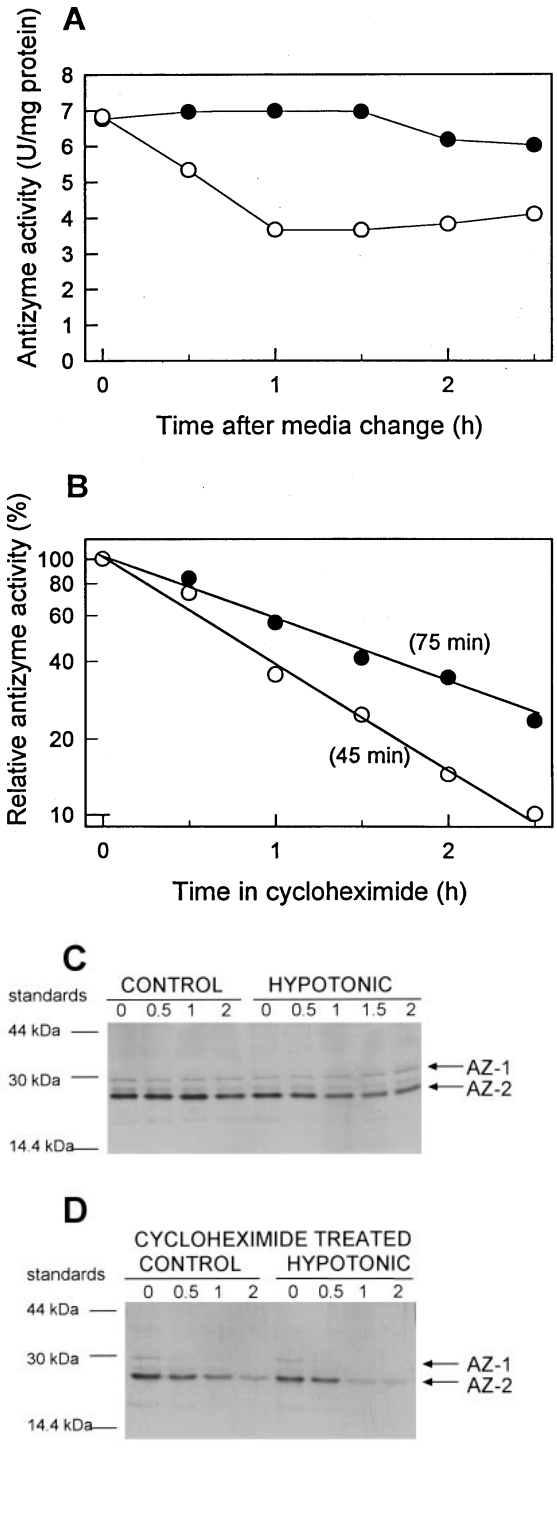

Figure 2 Antizyme stability changes associated with hypo-osmotic shock

HTC cell cultures were induced for antizyme as described in the legend to Figure 1, and subsequently some of the cells were diluted to 60% of the normal medium osmotic strength. (**A**) Change in antizyme activity in the diluted (○) and control (●) cultures. At the time of cell dilution, identical samples were also treated with 0.2 mM cycloheximide to inhibit further protein synthesis. (**B**) Decay of antizyme activity in these cultures. Antizyme protein changes in this experiment were evaluated by separating cell proteins by SDS/PAGE, blotting to nitrocellulose and immunologically detecting antizyme bands. (**C** and **D**) Changes in the antizyme bands that occur in correlation with the activity changes depicted in (**A**) and (**B**) respectively. U, units.

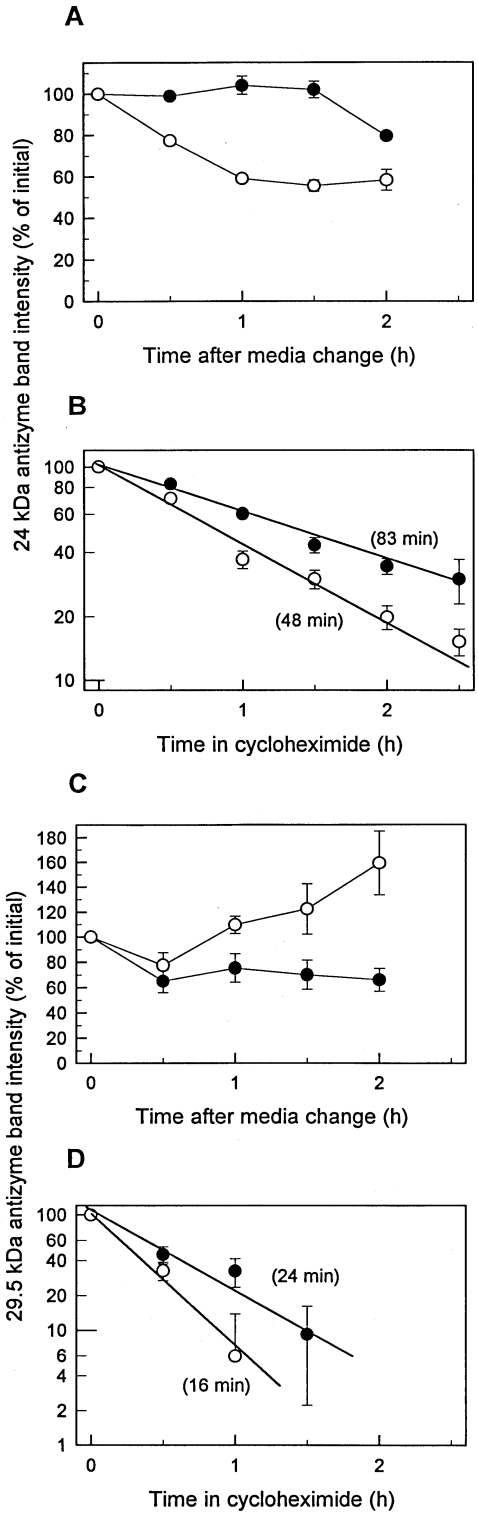

Figure 3 Analysis of changes in antizyme protein following hypo-osmotic shock

The relative intensities of the antizyme protein bands detected as shown in Figures 2(**C**) and 2(**D**) were evaluated by densitometric analysis. The means ± S.D. of multiple ($n \geqslant 3$) assays are plotted for samples from control (●) or diluted (○) cultures, in the absence (**A** and **C**) or presence (**B** and **D**) of cycloheximide. (**A** and **B**) Changes in the 24 kDa antizyme band (AZ-2); (**C** and **D**) variations in the 29.5 kDa band (AZ-1).

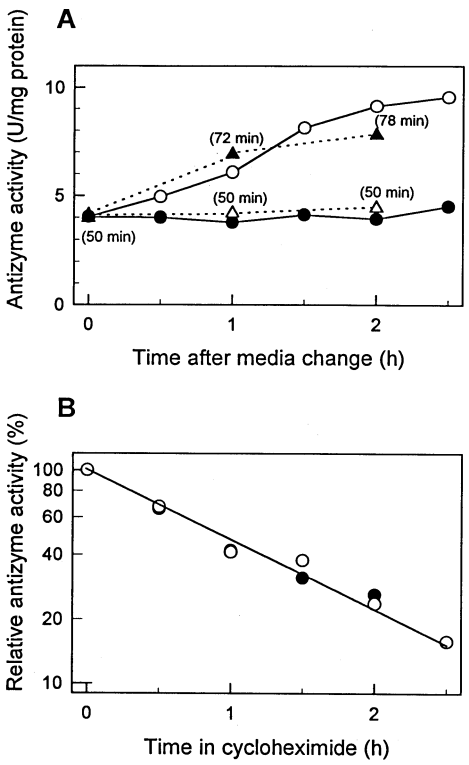

Figure 4 Reversal of hypo-osmotic shock

HTC cell cultures were induced for antizyme and then diluted to 60% of the isotonic medium, as for Figures 1–3. After 2 h at the reduced osmotic conditions, the culture was divided, and 100 mM NaCl was added to one of the aliquots. At this time ($t = 0$), each culture was divided, and cycloheximide was added to half. Samples were evaluated for changes in antizyme activity induced by the change from dilute to hyperosmotic conditions (○). Cells remaining in the hypo-osmotic conditions (●) served as the controls (**A**). The immediate effect of this abrupt increase in medium osmotic strength on the stability of antizyme activity is shown by the exponential decay curve in (**B**). Also shown in (**A**) is the result of an identical repeat of this experiment in which culture aliquots were treated with cycloheximide at $t = 1$ and 2 h as well as at $t = 0$. The antizyme activity half-lives for this second experiment are indicated in parentheses for the diluted (△) and hyperosmotic media cultures (▲). U, units.

was added to prevent further protein synthesis. As seen in Figure 2(B), there was an immediate decrease in the half-life of antizyme in the cells placed in the hypo-osmotic culture conditions. The reduction from a 75 min to a 45 min half-life for antizyme could account for most, if not all, of the decrease observed in the steady-state activity of this regulatory protein. It should be noted that this change in antizyme stability is immediate and, since it is observed in the presence of cycloheximide, does not appear to require synthesis of any additional protein factors.

Additional studies were performed to determine whether the response of antizyme to hypo-osmotic shock was due to changes in the level of antizyme protein rather than just inactivation of existing antizyme by post-translational modification. Cell protein samples were resolved by SDS/PAGE and blotting to nitrocellulose membranes, and antizyme protein was detected by binding of affinity-purified polyclonal antibody (Figures 2C and 2D). As noted previously [24], HTC cells express two prominent antizyme bands, of approx. 29.5 and 24 kDa. The larger, which represents about 10–20% of the total, is denoted as AZ-1, because it is the characteristic size of antizyme translated *in vitro* from the first AUG in the antizyme mRNA open reading frame (J. L. A. Mitchell and G. G. Judd, unpublished work). The more intense, smaller antizyme band (AZ-2) is thought to result from

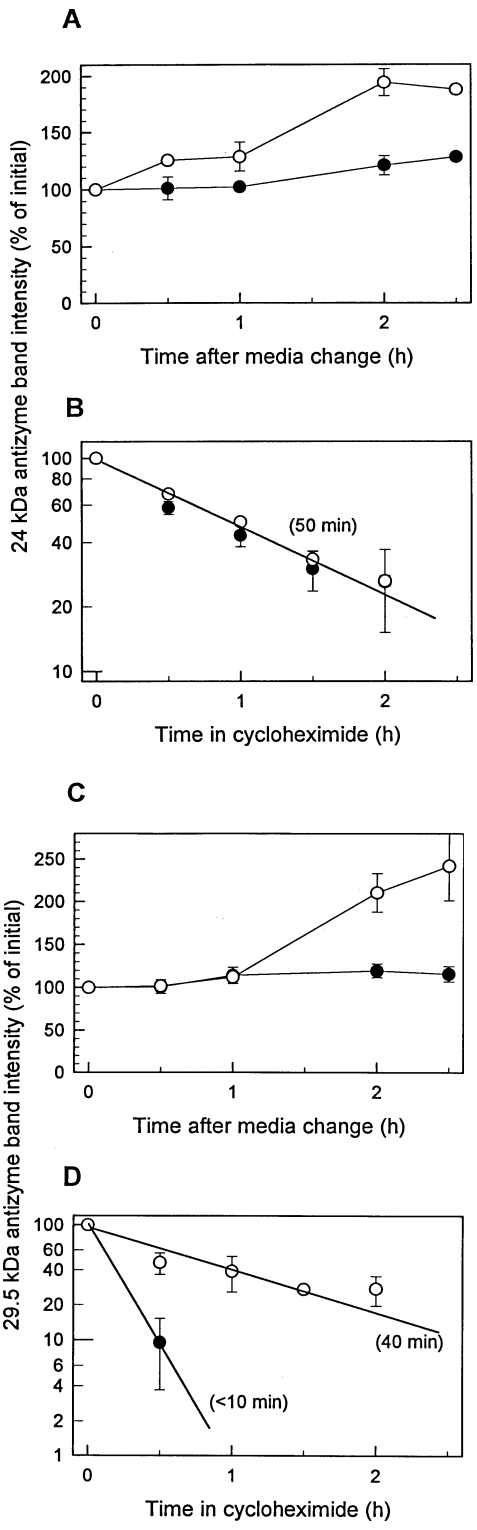

Figure 5 Analysis of changes in antizyme protein following hyperosmotic shock

Replicate samples from the experiment shown in Figure 4 were analysed for antizyme protein as described in the legends for Figures 2 and 3. The relative intensities of the antizyme protein bands are plotted as means ± S.D. of multiple ($n \geqslant 3$) assays for samples from control (continued hypo-osmotic media, ●) or hyperosmotic (○) cultures, in the absence (**A** and **C**) or presence (**B** and **D**) of cycloheximide. (**A** and **B**) Changes in the 24 kDa antizyme band (AZ-2); (**C** and **D**) variations in the 29.5 kDa band (AZ-1).

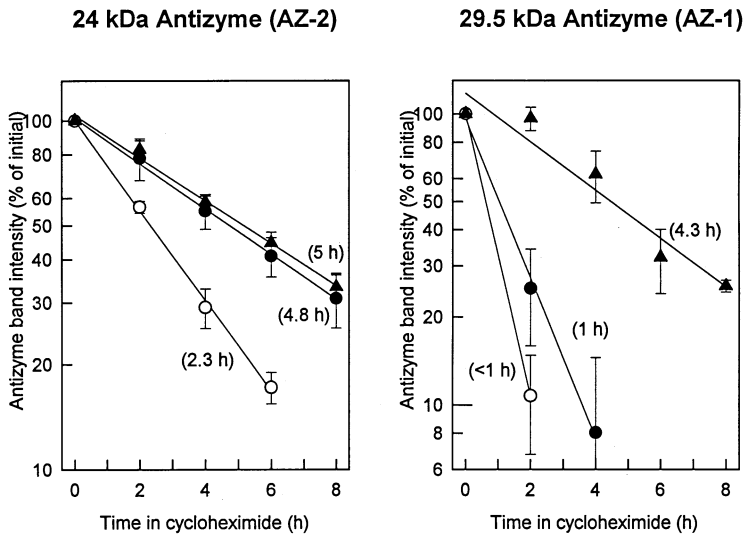

Figure 6 Effect of abrupt changes in medium osmolarity on antizyme protein stability in DH23b cells

Antizyme was induced in DH23b cells cultured with 4 mM difluoromethylornithine by the addition of 1 mM putrescine. After 4 h the medium was replaced with fresh medium (control; ●), medium diluted 50% with deionized water (hypotonic; ○), or medium containing 100 mM NaCl (hypertonic; ▲). Cycloheximide (0.2 mM) was added to each culture so that the stability of the antizyme forms could be evaluated. Samples were chromatographed by SDS/PAGE, and the major antizyme forms were identified and quantified as indicated in Figures 2 and 3. Changes in AZ-1 and AZ-2 are plotted as means ± S.D. for three replicate experiments.

translation that has initiated at the second AUG in the antizyme mRNA [22,23].

Replicate immunoblots, such as those shown in Figure 2, were scanned and quantified to illustrate more clearly the changes in relative intensities of the two antizyme forms in response to hypo-osmotic shock. As seen in Figures 3(A) and 3(B), the relative changes in AZ-2 (24 kDa band) closely paralleled the changes in antizyme activity noted in Figures 2(A) and 2(B). Since AZ-1 comprises only 10–20% of the total antizyme, its contribution to the measured antizyme activity is anticipated to be proportionally minor. Thus, although the changes in antizyme activity shown in Figure 2, as well as in the subsequent experiments, appear to reflect variations in the major antizyme band, AZ-2, they are in fact measurements of total antizyme activity. In this analysis the antizyme AZ-2 protein half-life appears to decrease from about 83 to 48 min. Surprisingly, the levels of AZ-1 do not follow the changes noted in AZ-2. This antizyme form actually increases somewhat following the transition to hypo-osmotic media (Figure 3C). Further, AZ-1 appears to be less stable than AZ-2: its half-life is estimated at about 24 min, compared with 83 min for AZ-2. Contrary to expectations from the results seen in Figure 3(C), AZ-1 does not increase in stability in response to this change in medium, and it may even become more labile. Unfortunately, the minute quantities of this antizyme form make it very difficult to determine this short half-life with great accuracy.

Antizyme response to the transition from hypo-osmotic to hyperosmotic conditions

The destabilization of antizyme and depression of antizyme levels, which were induced by hypo-osmotic culture conditions, were both reversed by increasing the osmotic strength of the medium. As shown in Figure 4, re-establishment of the longer half-life for antizyme was not immediate, and perhaps not even completed within 2 h. Further, this increase in antizyme stability appears to require new protein synthesis, as the samples exposed to cycloheximide at the time of increased tonicity did not show any evidence of alteration in the degradation rate for this protein during the course of the decay study (Figure 4B). As with the hypotonic shock experiments above, the antizyme activity changes induced by hypertonic shock were closely paralleled by variations in protein levels of the major antizyme form, AZ-2 (Figures 5A and 5B). Like the major antizyme form, AZ-1 also increased in concentration following hyperosmotic shock, but in this case there was an immediate increase in the apparent stability of this antizyme form, to a half-life of about 40 min. Under these hypertonic culture conditions the AZ-1 protein appears to be much more stable than it is in normal, isotonic media.

Osmotic stress also induces antizyme stability changes in a mutant cell line (DH23b) that overproduces both ODC and antizyme

There is nothing known about the mechanism of degradation of antizyme except that this degradation is somewhat impeded in cells that contain more-stable forms of ODC. We have shown previously that the ODC-overproducing HTC line DH23b accumulates a stable form of ODC protein to about 1000-fold normal levels, and antizyme levels in these cells are generally 10- to 50-fold greater than the parental cells [24]. This increase in antizyme appears to result from greatly enhanced stability of otherwise normal antizyme protein.

We studied the effect of osmotic shock on antizyme in this mutant line in an attempt to determine whether the mechanism of response to osmotic shock would be limited, and thereby ineffective, at these very high antizyme levels. As shown in Figure 6, the normal half-lives of both of the antizyme forms were much longer than observed in the parental HTC cells. In spite of these elevated antizyme concentrations, the responses of these forms to osmotic shock were qualitatively similar to that seen in the HTC cells. Hypo-osmotic shock induced an immediate destabilization

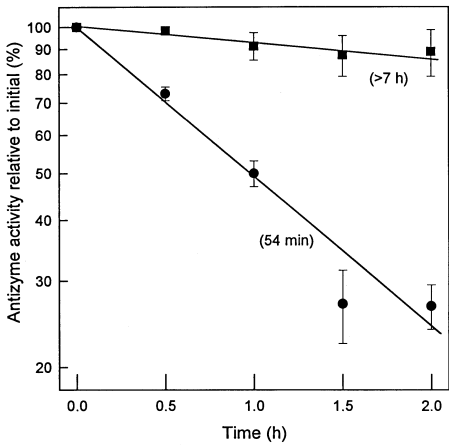

Figure 7 The energy dependence of antizyme degradation

Antizyme was induced in HTC cultures by the addition of 1 mM spermidine. After 4 h the cells were washed and eventually resuspended with pre-warmed PBS. Cycloheximide (0.2 mM) was added, and the culture was divided, with one half treated with 5 mM glucose (●) and the other with 5 mM 2-deoxyglucose/0.8 mM dinitrophenol (■). Shown are the means ± S.D. for three replicate experiments.

of the 24 kDa AZ-2 (a half-life change from about 5 h to 2.3 h), as well as an apparent destabilization of the 29.5 kDa AZ-1. As with the HTC cells, hyperosmotic shock did not immediately affect the AZ-2-form stability, but it did induce an immediate increase in the stability of the AZ-1 form of antizyme.

Antizyme degradation is an energy-dependent process

The absence of any information on the mechanism of degradation of antizyme severely restricts understanding of potential mechanisms whereby osmotic shock alters the stability of this critical protein. Many labile regulatory proteins that are critical to normal cell physiology are found to require specific energy-dependent proteolytic pathways for their rapid and controlled degradation. As shown in Figure 7, antizyme degradation also appears to require one or more energy-dependent steps. In this series of experiments, cells that were induced for antizyme were treated with cycloheximide in the presence of PBS containing either glucose or a combination of 2-deoxyglucose and dinitrophenol. The rapid loss of antizyme activity in the glucose controls was almost completely prevented by conditions that limit ATP production. Further, analysis of the specific antizyme forms in this experiment indicated that degradation of both AZ-1 and AZ-2 was blocked by this treatment (results not shown).

DISCUSSION

Cellular accommodations to osmotic stress have long been noted to involve changes in putrescine levels through increases in ODC synthesis and stability, and polyamine transport. In the present study we have provided evidence that all these polyamine-related changes can be explained by osmotic-stress-induced variations in the level of the regulatory protein antizyme. Further, we have demonstrated that these adjustments in cellular antizyme levels are attributed to alteration in the rate of antizyme degradation, a mechanism of antizyme regulation that has not been previously reported.

Recent reports of the multiple effects of antizyme on polyamine synthesis and transport have enhanced appreciation for this regulatory protein playing a pivotal role in the maintenance of cell polyamine levels [13–19]. Because antizyme synthesis itself is controlled by the cellular concentrations of spermidine and spermine, it is generally assumed that antizyme's function is restricted to maintenance of polyamine homoeostasis. Without invoking other controls, however, this proficient feedback system would prevent cells from adopting new steady-state polyamine levels in response to growth signals, differentiation or stress. Interestingly, there has been only one report of antizyme control at the level of transcription. Yang et al. [26] have observed that interleukin-1 stimulated transcription of the antizyme gene and effected ODC down-regulation in human melanoma cells. In most tissues, however, it appears that the level of antizyme mRNA is not limiting [27], and that the regulation of antizyme synthesis is controlled by the effect of the polyamines on the efficiency of the +1 frame-shift event required for translation of this message [22]. As such, transcriptional regulation of the antizyme gene may be of relatively minor importance. Conversely, as a labile protein, antizyme would be expected to respond rapidly to variations not only in synthesis but also in degradation. As we have now shown, osmotic stress does exert an immediate and proportional change in the stability of antizyme. This, in turn, results in the rapid achievement of a new steady-state level of cellular antizyme, without altering the feedback system of antizyme synthesis. Such a change in antizyme stability would be expected to provide a new set-point polyamine level for the polyamine homoeostatic control exerted at the level of antizyme synthesis. Thus, this new level of antizyme regulation may be responsible for the achievement and maintenance of different steady-state concentrations of cell polyamines.

Is a relatively small change in antizyme level sufficient to produce the large responses that have been reported in polyamine biosynthesis and transport? In the present experiments, a 40 % decrease in media osmolarity induced an approx. 50 % decrease in the steady-state level of antizyme, yet others have reported that a similar hypo-osmotic change resulted in a several-fold induction of ODC activity [10] or 3–6-fold increase in ODC half-life [9]. Two factors appear to explain this apparent disparity. First, in normal cells antizyme is constantly being produced and low levels can almost always be observed by immunodetection techniques, but rarely is it produced in excess of its high-affinity substrate, ODC. Thus the availability of this regulatory protein seems to be rate limiting. Secondly, antizyme is thought to act catalytically in the turnover of ODC protein [28,29], and, by extension, in the rapid degradation of nascent ODC during translation [15,16]. Thus there would be a natural amplification of response in reactions affected by adjustments in levels of the catalytic agent, antizyme.

Understanding of how osmotic stress, and perhaps other cellular signalling pathways, induces a change in the stability of antizyme protein first requires knowledge of the mechanism of this degradation. Unfortunately, studies of antizyme protein degradation have been very limited. Murakami et al. [30] indicated that antizyme activity undergoes rapid turnover, and that the stability of this activity depended upon the presence of bound ODC. Our past studies of immunologically detected AZ-2 confirmed that antizyme is normally a labile protein, and its stability is greatly enhanced when it is complexed with a mutant, stable, form of ODC [24]. Citing other experimental results, Hayashi and co-workers [13,29] speculated that some antizyme is probably lost in cycling ODC protein to degradation by the 26 S proteasome, yet such use-associated degradation was not confirmed in *in vitro* studies using an active antizyme fusion protein construct [13,31]. In the current investigation, we have established that antizyme degradation, like that of other labile regulatory proteins, is energy dependent. This suggests the requirement of a

specific proteolytic process, such as that involving the 26 S proteasome, rather than a non-specific protease sensitivity. Published reports [32], and ongoing studies in our lab, indicate that antizyme is not readily degraded in rabbit reticulocyte lysate, an *in vitro* system that normally promotes degradation of other proteins that are substrates for the 26 S proteasome. It may be that the initiation of antizyme degradation requires an additional, unidentified, factor, the same way that rapid ODC degradation requires the presence of antizyme. However, the immediate decrease in stability upon a shift to hypo-osmotic culture conditions minimizes the possibility that antizyme degradation is limited by the level of such a destabilizing factor. Conversely, the increase in antizyme stability upon hyperosmotic shock is not immediate, and does not occur in the presence of cycloheximide. This would be consistent with a requirement for new protein synthesis, such as a stabilization factor or new antizyme molecules, before greater antizyme stability was achieved. Clearly the mechanisms of antizyme degradation and any stabilizing or destabilizing factors must be elucidated before we can begin to understand the control of this process by osmotic stress.

The enhanced stability of antizyme in the ODC-overproducing mutant, DH23b, is consistent with a previous suggestion that the antizyme–ODC complex may protect antizyme from its normal degradation pathway [30]. It is unlikely, though, that this has any bearing on the control of antizyme stability by osmotic shock, as the same qualitative changes were noted under conditions of saturating ODC in the DH23b cells, and the complete absence of this enzyme in polyamine-fed HTC cells. Further, whatever change is involved in the antizyme-degradation pathway must not require a factor produced in limited quantities, as this would have resulted in minimal variation in half-life in the high antizyme concentrations found in DH23b cells. Instead, the results using DH23b cells are more consistent with a change in the antizyme protein itself being the rate-limiting event in antizyme degradation that is modified by varying medium osmolarity.

One of the most surprising discoveries in these studies was the observation that the two major forms of antizyme protein were not affected equivalently by osmotic stress. Cellular levels of the major antizyme protein (AZ-2), which is thought to be the product of the second translation start site in the antizyme mRNA, vary in concert with the observed changes in antizyme activity. In contrast, the minor antizyme protein (AZ-1) exhibits a much shorter half-life, and its stability was immediately affected by hyperosmotic shock, as opposed to the delayed response seen with AZ-2. That AZ-1 is really the product of translation initiated at the first in-frame AUG of the antizyme mRNA is supported by the following observations. The band (a) co-migrates on SDS/PAGE with antizyme synthesized *in vitro* from a P-cite vector that promotes almost exclusive initiation at this initial start site; (b) only appears in polyamine-treated cells; (c) is recognized by affinity-purified polyclonal antibody made against antizyme produced in bacteria; and (d) is overproduced in DH23b cells along with the main antizyme band. The differential responsiveness of these two forms of the same protein could suggest many interesting possibilities. For example, it may be that the N-terminal peptide, which is missing in AZ-2, is a factor in the recognition of antizyme during its proteolytic degradation, or that these two antizyme forms are sequestered in different regions of the cell. Associated with the noted differences in response to osmotic shock, it is not unlikely that these forms have distinct targets and activities in cells, a possibility of profound significance to considerations of the control of cellular polyamine metabolism.

These studies have greatly extended our understanding of how a cell can use the common regulatory intermediate, antizyme, to respond to the stress of osmotic shock with a co-ordinated change in several distinct processes involved in the regulation of cellular polyamine levels. The precise mechanism whereby antizyme stability is altered by the changed ionic environment of an osmotically stressed cell, and the differential role of the two antizyme forms in this response, are subjects of ongoing investigations.

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