Regulatory Properties of an Inducible Aliphatic Amidase in a Thermophilic *Bacillus*

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SUMMARY

A thermophilic bacillus growing on acetamide as both carbon and nitrogen sources produces an inducible amidase. This amidase hydrolysed the following amides in decreasing order of activity, in comparison with acetamide (1.00): propionamide (0.97), fluoroacetamide (0.84), formamide (0.35) and glycineamide (0.12). Cyanoacetamide, dimethylacetamide, dimethylformamide and urea also induced the synthesis of the amidase, but were not substrates of the enzyme. Studies with protoplasts suggest that the amidase is located in the cytoplasm.

Glucose strongly inhibited amidase synthesis; and limiting nitrogen did not release this inhibition. Urea strongly inhibited amidase activity in a competitive manner; but the inhibition caused by iodoacetamide and cyanoacetamide was non-competitive. Both thioacetamide and thiourea were effective inhibitors of enzyme induction.

Bacteria grown on a succinate-minimal medium exhibited a lag in amidase synthesis, which could be eliminated by decreasing the concentration of succinate. Acetate- or pyruvate-grown cultures behaved similarly, while those grown on alanine or glutamate exhibited no lag in enzyme induction.

In the mutant strain E21, repression of amidase synthesis by glucose was much less evident and no lag for induction was apparent with any of the other carbon sources mentioned.

INTRODUCTION

Induction of an aliphatic amidase by the corresponding amide was first found in *Candida utilis* (Clarke, 1970) grown on acetamide. In both *Pseudomonas aeruginosa* (Clarke & Houldsworth, 1968) and *Aspergillus nidulans* (Hynes, 1970) the inducible amidases are subject to catabolite repression. In the latter organism, amidase synthesis was also repressed by ammonia, the degree of repression being dependent on the carbon source (Hynes, 1970). An interesting relation between carbon and nitrogen metabolism of a catabolite-repressed enzyme was reported in *Klebsiella aerogenes* (Prival & Magasanik, 1971): in this case, nitrogen limitation relieved the catabolite repression of histidase and proline oxidase.

Thermophilic bacteria are of primary interest in their ability to grow at high temperatures. This ability is thought to be attributable to thermostable macromolecules (Farrell & Campbell, 1969). Although the thermostable character of many isolated cellular components of thermophilic bacteria has been well documented (Farrell & Campbell, 1969; Friedman & Weinstein, 1966; Marmur, 1960; Singleton & Amelunxen, 1973; Zeikos, Taylor & Brock, 1970), regulation of the synthesis of these components has not been explored.

The Jacob & Monod (1961) model of enzyme induction assumes a protein repressor molecule is inactivated by the inducer. Such a repressor molecule would be thermolabile

compared with proteins of thermophiles. The repressor in a thermophilic bacterium would have to be a thermostable molecule, at least under *in vivo* conditions.

Since enzyme regulation is a major aspect of cellular economy it is important to find out whether thermophilic enzymes are regulated in the same manner as mesophilic enzymes. Thermophily could be regarded as a more primitive condition in terms of evolution (Brock, 1967), in which case one would expect a less-developed system of regulation. On the other hand, it is also possible that thermophily developed from mesophily as an evolutionary adaptation under special ecological conditions. In the latter case, one would not expect any major differences in the regulatory control.

The object of this study was to investigate the regulatory control of the inducible amidase in a thermophilic bacillus.

METHODS

Bacterial strain and media. The thermophilic micro-organism and its properties, and also the minimal medium used, were described by Epstein & Grossowicz (1969). Sodium succinate (0.5 %) was substituted for glucose, unless otherwise indicated. A rich medium, trypticase soy broth (Difco), was also employed.

Growth. Cultures were grown at 55 °C with shaking in 100 and 250 ml Erlenmeyer flasks, containing 20 and 50 ml medium, respectively. The flasks had side arms to allow for turbidimetric measurements during growth. Growth was measured in a Klett colorimeter using filters no. 42 and 66 for minimal and rich media, respectively. A reading of 340 Klett units was equivalent to 0.5 mg dry weight/ml of the thermophilic organism in minimal medium.

Screening for inducers of amidase activity. Cells were grown overnight with the amides (30 mM). Substances which induced amidase activity were added to exponentially-growing cultures with an initial cell density of 30 to 50 Klett units.

Preparation of cells for enzyme assay. Samples of cultures were washed by centrifuging with 0.05 M-Tris-HCl buffer (pH 8.2); and resuspended to give a concentration of 0.5 mg dry weight/ml.

Preparation of cell-free extracts. Bacterial cultures were harvested and resuspended to give a concentration of 1 mg dry weight/ml. They were incubated at 37 °C for 30 min with lysozyme (250 µg ml⁻¹), centrifuged at 12000g for 15 min, and the supernatant fluid was used as the enzyme source. Protein was determined by the method of Lowry *et al.* (1951).

Amidase assay. Substrate solutions were prepared at a concentration of 200 µmol ml⁻¹ in 0.05 M-Tris-HCl buffer (pH 8.2). The enzyme source and the substrate (acetamide, unless otherwise indicated) were both pre-incubated separately at 55 °C for 10 min. The substrate was then added to the bacterial suspension (0.5 mg dry weight) or to the extract (0.5 mg protein), and the reaction mixture was made up to 2 ml with buffer. After incubation for 10 min, the reaction was stopped by adding 1 ml trichloroacetic acid (15 %) and the mixture was filtered through Whatman no. 50 filter paper. Release of NH₄⁺ was measured in the protein-free filtrate by the method of Muftic (1964) which is suitable for NH₄⁺ concentrations of 0.1 to 1.0 µmol ml⁻¹. At the enzyme concentration used, assays were linear for at least 30 min, and saturation was reached with 50 µmol substrate.

Transferase assay. A qualitative microassay was used (Clarke, 1972). The substrate mixture consisted of equal parts of amide (0.4 M) and freshly neutralized hydroxylamine hydrochloride (2 M) to two parts of 0.1 M-Tris buffer pH 7.2. The reaction mixture (0.2 ml substrate mixture and 0.1 mg dry weight bacterial suspension) was incubated for 10 min at 55 °C, and then the reaction was stopped by adding 0.4 ml FeCl₃ (60 %, w/v in 57 ml HCl made

up to 1 l with distilled water). Positive reactions (formation of a red-brown colour with an absorption peak at 540 nm) were compared with the colour formed by acetamide as substrate.

Preparation of protoplasts. Cells from the late-exponential growth phase were harvested and resuspended in 0.5 M-lactose in 0.05 M-Tris-HCl buffer (pH 8), incubated with lysozyme ($250 \mu\text{g ml}^{-1}$) for 30 min at 37 °C and centrifuged at 30000g for 15 min. When required, protoplasts were burst by resuspending in hypotonic buffer. The protoplast suspension was incubated for 15 min with cetyltrimethylammonium bromide (CTAB) to release membrane-bound amidase.

Isolation of mutants resistant to catabolite repression. The wild type was grown overnight in rich medium, washed, resuspended in minimal medium and u.v.-irradiated for 2.5 min at a distance of 50 cm. The cells were then grown for several generations in glucose-formamide (10 mM) minimal medium, in which the amide was the sole source of nitrogen. Samples were plated on glucose-formamide solid medium, and large colonies, on a lawn of pinpoint parent colonies, were picked and restreaked several times for purity.

Chemicals. Amides used were: benzamide, dimethylacetamide, dimethylformamide, formamide, *N*-methylacetamide and urea (all from BDH); cyanoacetamide, glycineamide hydrochloride and propionamide (Fluka AG, Buchs, Switzerland); nicotinamide (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); acetamide and thiourea (Riedel-DeHaen, AG, Sulze-Hannover, Germany); iodoacetamide and thioacetamide (Sigma); and fluoroacetamide (E. D. Bergmann, Hebrew University, Jerusalem).

RESULTS

Amidase induction

The thermophilic bacillus synthesized an inducible amidase when grown in minimal medium containing acetamide, and so various other amides and amide analogues were tested for their ability to induce amidase activity. Screening for enzyme inducers was carried out in succinate plus NH_4Cl medium so that the amides were not essential as carbon or nitrogen sources. Amidase activity was measured in whole cells after 3 h growth.

All amides which served as inducers were effective at a concentration of 30 mM. Acetamide and cyanoacetamide induced enzyme formation at a concentration of 10 mM; and formamide and urea (the only non-amide inducer discovered) were effective at concentrations as low as 5 mM (Table 1).

Substrate specificity

Pre-induced cells were used to test various acetamide analogues (at concentrations of 100 mM) as substrates of the enzyme. All amides which were substrates of amidase activity were also effective as inducers of the enzyme (Table 2). However, several amides were effective as inducers, but not as substrates (cyanoacetamide, dimethylacetamide, dimethylformamide and urea).

Inhibitors of enzyme activity

Amide analogues which did not serve as substrates of amidase activity, regardless of their effectiveness as inducers, were tested as possible inhibitors of enzyme activity. The assays using acetamide as substrate were carried out on cell-free extracts to eliminate permeability problems: these were prepared as described in Methods. Of the analogues tested, iodoacetamide inhibited amidase activity non-competitively, and a similar but weaker inhibition was obtained with cyanoacetamide. However, urea strongly inhibited enzyme activity in a competitive manner (Fig. 1 and Table 3).

Table 1. *Comparative activity of various amides as inducers of amidase activity*

Amide	Acetamide hydrolysed (μ mol/mg dry wt/min)	Induction (compared with acetamide)
None	0.01	—
Acetamide	7.73	1.0
Glycinamide	10.74	1.4
Formamide	10.44	1.4
Propionamide	9.81	1.3
Urea	8.38	1.1
Cyanoacetamide	7.53	1.0
Dimethylformamide	7.29	0.9
Fluoroacetamide*	6.33	0.8
Dimethylacetamide	6.23	0.8
<i>N</i> -valeramide	2.68	0.3
<i>N</i> -methylacetamide	1.24	0.2
Butyramide	1.00	0.1
Thioacetamide	0.01	—
Thiourea	0.01	—
Benzamide	0.01	—
Nicotinamide	0.01	—

* Fluoroacetamide inhibited growth after one generation.

Table 2. *Comparison of different amides as substrates*

Amide	Amide hydrolysed (μ mol/mg dry wt/min)	Activity (compared with acetamide)
Acetamide	8.64	1.00
Propionamide	8.38	0.97
Fluoroacetamide	7.26	0.84
Formamide	3.06	0.35
Glycinamide	1.02	0.12
Butyramide	0.12	0.01
Benzamide	0.12	0.01
Nicotinamide	0.01	—
<i>N</i> -valeramide	0.01	—
Cyanoacetamide	0.01	—
Thioacetamide	0.01	—
Urea	0.01	—
Thiourea	0.01	—
Dimethylformamide*		Inactive
Dimethylacetamide*		Inactive
<i>N</i> -methylacetamide*		Inactive

* Substituted amides were tested by the transferase assay as described in Methods.

Kinetics of induction

With low concentrations of formamide as inducer there was a lag of more than one generation before reaching maximal enzyme synthesis. Increasing concentrations of formamide decreased this lag, but inhibited growth. Since neither urea nor cyanoacetamide affected growth of the bacteria, these were used in preference to formamide for studying the kinetics of induction.

Fig. 1

Fig. 2

Fig. 1. Lineweaver-Burk plot of amidase activity. Cells pre-induced with formamide were tested using, as substrate: ●, acetamide; ○, acetamide + urea (5 mM); Δ, acetamide + cyanoacetamide (200 mM); and □, acetamide + iodoacetamide (15 mM). Concentrations causing 50 % inhibition were used. The amidase assay was as described in Methods.

Fig. 2. Kinetics of amidase induction using various concentrations of cyanoacetamide. An exponential culture growing in minimal medium was divided into several portions and grown with various concentrations of cyanoacetamide as inducer. Cells were harvested at intervals and enzyme activity and dry weight were determined as described in Methods. Concentrations of cyanoacetamide: ○, 1 mM; Δ, 10 mM; □, 100 mM.

Table 3. Kinetic parameters of acetamidase

Inhibitor	K_m (M)	V_{max} ($\mu\text{mol/mg}$ dry wt/min)
None	1.5×10^{-2}	16.67
Urea (5 mM)	7.1×10^{-2}	14.28
Iodoacetamide (15 mM)	1.6×10^{-2}	3.45
Cyanoacetamide (200 mM)	1.3×10^{-2}	6.41

With both urea and cyanoacetamide, there was a time lag before reaching the maximal rate of enzyme synthesis. This lag was not concentration dependent. There appeared to be a minimal concentration required (urea 5 mM, cyanoacetamide 10 mM), below which, no amidase was formed, and above which, the concentration had no effect on the rate of synthesis (Fig. 2).

Inhibitors of induction

Thioacetamide and thiourea inhibited the induction of amidase activity, 50 % inhibition being obtained using 7 mM-thioacetamide, or 18 mM-thiourea (Fig. 3). Inhibition of induction was similar, regardless of whether the inducer was also a substrate (acetamide) or not (cyanoacetamide, urea).

To inhibit induction, the analogue had to be added before enzyme synthesis began (Fig. 4). Thus, thioacetamide and thiourea seem to inhibit induction rather than affect amidase activity. Inhibition of induction was independent of inducer concentration.

Fig. 3. Inhibition of induction by amide analogues. Thioacetamide, ○, and thiourea, △, were added to exponentially growing cells in the presence of urea (10 mM) as inducer. Cells were harvested and enzymic activity was determined as in Fig. 2.

Fig. 4. Effect of time of addition of thiourea on inhibition of induction. Thiourea (20 mM) was added to exponentially growing cells at various time intervals after the addition of inducer. (a) ○, no thiourea, △, thiourea added simultaneously with the inducer, (b) thiourea added after inducer at times indicated by arrows. Enzyme activity was determined as in Fig. 2.

Table 4. Comparison of enzyme activity in different cell preparations

Source of enzyme	Amidase activity ($\mu\text{mol}/\text{mg}$ protein/min)
Whole cells*	11.5
Cell-free extract	16.3
Protoplast supernatant	0.1
Burst protoplasts	14.7

* mg dry wt.

Localization of amidase

Similar activities were obtained with whole cells and cell-free extracts. Since many catabolic enzymes are located in the periplasmic space between the wall and the cytoplasmic membrane (Neu & Heppel, 1964), this might be the site of amidase activity. If this assumption is correct, amidase activity should be found in the supernatant fluid of protoplasts.

Protoplasts were prepared from pre-induced cells as described in Methods; and enzyme activity was measured in the supernatant fluid and in the protoplasts after they were burst in hypotonic buffer (Table 4).

No enzyme activity was found in the periplasmic space, but amidase activity could be bound to the membrane. To investigate this, protoplasts were incubated with CTAB at

Table 5. *Enzyme activity of protoplasts treated with various concentrations of CTAB*

Activity was determined in the supernatant after incubation of the protoplast suspension for 15 min at 37 °C with increasing concentrations of CTAB.

CTAB ($\mu\text{mol/ml}$)	Extinction (Klett units: filter no. 42)	Absorption at 260 nm	Enzyme activity ($\mu\text{mol/mg}$ protein/min)
None	2000	0.406	—
0.001	2000	0.429	—
0.005	2000	0.451	—
0.01	1875	0.396	—
0.05	1900	0.417	—
0.1	1825	0.505	8.58
0.5	1700	0.892	11.61
1.0	1400	0.827	13.68

Fig. 5. Synthesis of amidase by cells grown on glucose-acetamide minimal medium. The wild type was grown on glucose (0.02 %) and acetamide (10 mM); samples were taken for determination of ○, growth (dry weight) and Δ , enzyme activity.

concentrations that were too low to cause lysis, yet sufficient to release membrane-bound enzyme. The effect of CTAB was determined by a decrease in turbidity and an increase in absorption at 260 nm. Lysis of the protoplasts occurred at CTAB concentrations of $1.0 \mu\text{mol ml}^{-1}$ and above. Therefore, concentrations between 0.001 and $1.0 \mu\text{mol ml}^{-1}$ were used to determine whether or not appearance of enzyme activity in the supernatant fluid preceded the appearance of lysis. No amidase activity appeared in the supernatant fluid until the protoplasts showed leakage as determined by an increase in absorption at 260 nm (Table 5). Thus, we assume that the enzyme is cytoplasmic.

Effect of carbon source on enzyme induction

Bacteria growing in minimal medium with glucose as the carbon source showed no measurable enzyme activity with any of the inducer amides. Cells grown on limiting glucose (0.02 %) plus acetamide (10 mM) showed a diauxic growth curve. As long as the glucose concentration was sufficient to support growth, the growth rate was typical of that obtained on glucose alone. When the glucose supply was exhausted, growth ceased and was subsequently resumed on acetamide at a lower growth rate, characteristic of the amide. Amidase activity began to appear when the glucose was exhausted (Fig. 5).

When succinate was used as the carbon source, amidase formation occurred, but only

Fig. 6. (a) Growth and (b) amidase synthesis on succinate and acetamide minimal medium. The wild type was grown on various concentrations of succinate-acetamide (10 mM) minimal medium: ●, no succinate; ○, 0.05 %; Δ, 0.1 %; and □, 0.5 % succinate.

Fig. 7

Fig. 8

Fig. 7. Kinetics of induction on various carbon sources. Wild-type cells were grown on various carbon sources in presence of urea (30 mM) as inducer; ●, alanine 0.25 %; ▲, glutamate 0.25 %; Δ, pyruvate 0.25 %; ○, acetate 0.25 %. Enzyme activity was determined as in Fig. 2.

Fig. 8. Synthesis of amidase by mutant strain E21. The strain was grown overnight like the wild type and enzyme activity determined as in Fig. 6; ○, succinate-grown cells; Δ, glucose-grown cells.

after a distinct time lag. Using limiting concentrations of succinate plus 10 mM-acetamide, a diauxic growth was obtained. The point at which growth stopped, presumably due to succinate exhaustion, coincided with the point of maximal enzyme synthesis (Fig. 6). Decreasing concentrations of succinate diminished the lag in enzyme synthesis. Cells grown on acetamide and ammonia, without added succinate, exhibited no lag.

Bacteria grown on pyruvate or acetate as carbon sources showed a lag in the synthesis of amidase similar to that caused by growth on succinate. However, cells grown on alanine or glutamate showed no apparent lag (Fig. 7).

Isolation of mutants resistant to catabolite repression

Mutant strains were isolated on minimal medium containing glucose and formamide (see Methods). Since formamide is a good inducer but a poor substrate, only those cells which can make a lot of amidase in the presence of glucose will grow well and form large colonies. Strain E21 was isolated as a large colony on a lawn of pinpoint parent colonies. The mutant, unlike the parent strain, synthesized enzyme on minimal medium with either glucose or succinate without any lag; the synthesis was insensitive to varying concentrations of succinate (Fig. 8). The mutant synthesized more enzyme than the wild type. The kinetics of enzyme activity were also slightly different; a double reciprocal plot showed a somewhat higher apparent K_m value (8.3×10^{-2} M) and a higher V_{max} (45.91) for the enzyme from the mutant strain compared with the wild type.

Effect of nitrogen starvation on catabolite repression

Nitrogen starvation caused an escape from catabolite repression (Dover & Halpern, 1972; Hynes, 1970; Prival & Magasanik, 1971), so we investigated the response of the thermophilic bacillus under conditions of limiting nitrogen. No growth of the organism could be obtained on glucose-acetamide medium without an added source of nitrogen. When succinate was substituted for glucose, growth was obtained; but the kinetics of enzyme induction were identical to those in succinate-acetamide medium containing NH_4Cl . The mutant strain also showed no release from catabolite repression under conditions of nitrogen starvation.

DISCUSSION

Inducible amidase synthesis by a thermophilic bacillus is specific for aliphatic amides; aryl amides act neither as substrates nor as inducers. This is consistent with results for mesophilic organisms (Kelly & Clarke, 1962).

Inducer action differed from substrate action; thus cyanoacetamide and urea serve as inducers but not as substrates. Urea is also a strong competitive inhibitor of enzyme activity, while cyanoacetamide inhibits enzyme activity less strongly and in a non-competitive fashion. Though different in their inhibitive effect, these two inhibitors seem to induce enzyme synthesis in a similar manner.

The presence of a time lag with both substrate inducers and non-substrate inducers, before maximal amidase synthesis occurs, is consistent with results for *Pseudomonas* (Kelly & Clarke, 1962) and may be a result of catabolite repression. The following evidence supports this hypothesis: (i) carbon-starved bacteria showed no lag in amidase formation. (ii) Growth on succinate, unlike growth on glucose, allowed enzyme synthesis, but only after a distinct lag, which decreased with a decrease in succinate concentration; a similar lag occurred in cells grown on acetate or pyruvate. (iii) No lag in amidase formation occurred when the cells were grown on alanine or glutamate. (iv) The mutant strain which is more resistant to catabolite repression than the wild type exhibited no lag in amidase synthesis. (v) Diauxic growth was obtained in glucose-acetamide-ammonium medium.

When the internal concentration of catabolites is high as a result of growth on glucose, catabolite repression is severe. It is minimal when the pool of catabolites is depleted as in carbon starvation. Between these two extremes there is probably a weak repression of amidase synthesis as in growth on succinate, pyruvate and acetate.

The rather high concentration of inducer (10^{-2} M) which was required for synthesis suggests either some permeability barrier or lack of a concentrating mechanism of the amides

within the cell. Urea and cyanoacetamide serve as inducers and are also effective inhibitors of enzyme activity. These substances can be washed out easily during harvesting of the cells, suggesting that they are not bound intracellularly. Thus, the lag could be explained as the time required for sufficient quantities of inducer to accumulate within the cell. However, above a minimal concentration (10 mM-cyanoacetamide or 5 mM-urea), increasing the concentration of the non-substrate inducer did not decrease the lag. This would seem to discount the effect of permeability.

Nitrogen starvation did not alleviate the catabolite repression of enzyme synthesis, in contrast to the enzymes of the γ -aminobutyrate pathway in *Escherichia coli* K12 (Dover & Halpern, 1972), acetamidase in *Aspergillus nidulans* (Hynes, 1970) and histidase in *Klebsiella aerogenes* (Prival & Magasanik, 1971), in which nitrogen limitation was found to release the cell from catabolite repression.

Thus, the mechanism operating in the thermophile seems to be a 'less-fine control', which puts the organism at a selective disadvantage as it is unable to utilize acetamide as a nitrogen source in the presence of glucose or high concentrations of succinate. If thermophily is a more primitive condition in terms of evolution, one would expect to find a more primitive system of regulation in the thermophilic bacillus. However, this lack of 'fine control' between carbon and nitrogen metabolism is not exclusive to thermophilic bacteria. A similar condition has been reported for the histidase, a catabolic enzyme, in *Bacillus subtilis* (Chasin & Magasanik, 1969) in contrast to that in *K. aerogenes*. Thus, the results obtained are not necessarily characteristic of thermophilic micro-organisms.

Although certain regulatory properties of the thermophilic bacteria are being unravelled, as yet, we do not know of any regulatory characteristics which are specific for thermophiles.

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