THE EFFECT OF IMIDOESTERS ON THE PROTOCATECHUATE 3,4-DIOXYGENASE ACTIVITY OF *ACINETOBACTER CALCOACETICUS*

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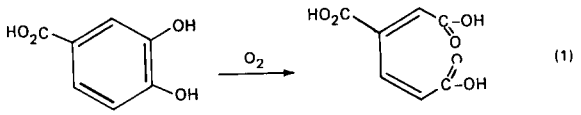
1. Introduction

Protocatechuate 3,4-dioxygenase, PCD, (EC 1.13.1.3) is a non-heme trivalent iron-containing enzyme that catalyzes the transformation of protocatechuic acid, PCA, to β-carboxy-*cis*, *cis*-muconic acid (eq. 1)



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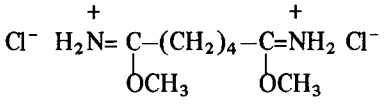
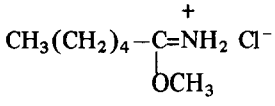
has been isolated from several bacterial sources, and the enzyme from *Pseudomonas aeruginosa* has been crystallized and immobilized [1]. In our continuing studies of this dioxygenase, the bacteria from which we obtain it, and the general field of enzyme immobilization, we initated a study of the effect of the bifunctional imidoester, dimethyl adipimidate, DMA, and the corresponding monofunctional reagent, methyl hexanoimidate, MHI, on the catalytic and structural properties of purified PCD, crude



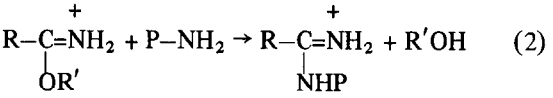
Dimethyl Adipimidate, DMA



Methyl Hexanoimidate, MHI

extracts, and whole cells of *Acinetobacter calcoaceticus*.

A decided advantage of using the imidoester functionality for modifying proteins or other amino-containing macromolecules in pure or crude, soluble or insoluble form, it its high chemical selectivity. With proteins, imidoesters react only with the therminal α-amino group and the ε-amino groups of lysyl residues to form amidines (eq. 2). The resulting amidinated proteins exhibit negligible changes in their conformation and retain the same net charge. The use of imidoesters for



(2)

protein stabilization and immobilization has been the subject of previous reports [2–4].

We now report initial results on the effect of DMA and MHI on the activity and viability of whole cells. Studies on the chemical modification of whole cells have been reported by others, but with reagents less selective than imidoesters [5–7].

2. Materials and methods

A. calcoaceticus 80-1, a derivative of ATCC14987, was grown in P_1 minimal media supplemented with 0.5% PCA [8]. Cells were harvested in late log phase, washed twice with 50 mM Tris–HCl–0.85% NaCl buffer, pH 7.5 and resuspended. Cell suspensions were stable for at least 72 hr at 5°C in terms of viability and PCD activity.

Crude enzyme extracts were prepared by sonication of washed cell suspensions (2 min at maximal output

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of a Megason ultrasonic disintegrator) followed by centrifugation at 50 000 *g* for 20 min. The supernatant solution was used as the crude enzyme.

The spread plate method, using nutrient agar, was used to determine cell viability. Oxygen consumption was measured at 25°C using a Yellow Springs Instrument Model 53 oxygen monitor. PCD activity was measured by PCA consumption as previously described [1]. Methyl imidoesters were prepared from the corresponding nitriles and a methanol–HCl solution [9].

Modification of whole cells and enzyme solutions was conducted in buffer at pH 8.0 and 25°C by adding portionwise the solid imidoester hydrochloride salts followed by careful readjustment of the pH to 8.0 with 1 N NaOH. After addition of DMA or MHI, samples were periodically withdrawn and analyzed for PCD activity (decrease in absorbance at 290 nm), oxygen consumption and viability.

Equivalent imidoester functionality concentrations (1.0% wt/vol) of DMA and MHI were employed (i.e., 20 mg MHI or 10 mg DMA/2.0 ml of cell suspension containing ca. 3 mg, dry wt, of cells). The control, a cell suspension at pH 8.0 containing no imidoester, was concurrently examined for activity and viability.

3. Results and discussion

Fig. 1 shows the effect of DMA and MHI on cell viability and PCA and O_2 consumption. Upon addition of DMA, there is an immediate decrease in the PCA uptake (from 100% to 82%), a decrease in oxygen consumption (60%), but no apparent decrease in cell viability. After 1 hr, the PCA uptake is zero, the O_2 consumption is 13.7% that of the original cell suspension, and the cells are not viable. At 2 hr, the O_2 uptake is 1.8%.

Addition of the monofunctional reagent MHI to the cell suspension results in a similar initial decrease in PCA uptake (from 100 to 76%) and in O_2 consumption (28%), but an immediate loss of viability. However, after this initial decrease in PCA and O_2 consumption, there is no further reduction in these activities. PCA uptake remains at approx. 60% and O_2 utilization remains at approx. 30% relative to their initial values. Cells not treated with DMA or MHI retain their full activities and viability for the same time period at 25°C.

To determine whether this effect of DMA is limited to whole cells, a crude soluble fraction of *A. calcoaceticus* was treated with the bifunctional reagent. No change in PCD activity was observed upon addition of DMA, either immediately or after 2 hr. Similarly, the control showed no change in activity during the same time period. Both the DMA-treated supernatant and the control retained similar activity with time (approx. 80%) even after 19 hr at 25°C. Furthermore, *pure* PCD, obtained from *P. aeruginosa,* treated with DMA exhibited no reduction in activity.

Imidoesters react selectively with primary amino groups of proteins and with certain other amino-containing substances. Because DMA had no effect on the activity of pure *P. aeruginosa* PCD or *A. calcoaceticus* cell free extracts (these enzymes being perhaps identical), it is reasonable to assume that there are no primary amino groups in this enzyme(s) that are essential for catalysis or substrate binding (see ref. [9] which describes a system in which an amino group is involved in substrate binding). The complete loss of activity in the DMA-treated cells, as opposed to only a partial reduction in activity in the MHI-treated cells, indicates that the effect is due to the nature of the particular reagent (bifunctional vs. monofunctional) and not due to the imidoester functionality, per se. Also, loss of activity is not due to enzyme inactivation. Although not unequivocably established, the reduction in activity (both PCA and O_2 consumption) can be attributed to intramolecular crosslinking of the cell wall or membrane components with the bifunctional reagent. Intramolecular crosslinking could 'tighten' the cell wall and thereby prevent ready access of PCA and O_2 to PCD. On the other hand, although MHI also kills the cells, the monofunctional reagent cannot cause crosslinking and hence does not prevent total transfer of PCA and O_2 to the cell-bound enzyme. In support of this working hypothesis, sonication of 'killed and inactive' DMA-treated cells produced a particulate and soluble fraction, both of which exhibited substantial activity. Microscopic examination of the DMA-treated cells revealed no aggregates, hence no apparent intermolecular cros linking. Some aberrant cell forms (pear-shaped instead of rod-shaped) were observed with DMA as well as with MHI. Neither DMA nor MHI caused any disruption of the cells as evidenced by finding no enzyme activity in filtrates of treated cell suspensions. The absence of intermolecular cross-

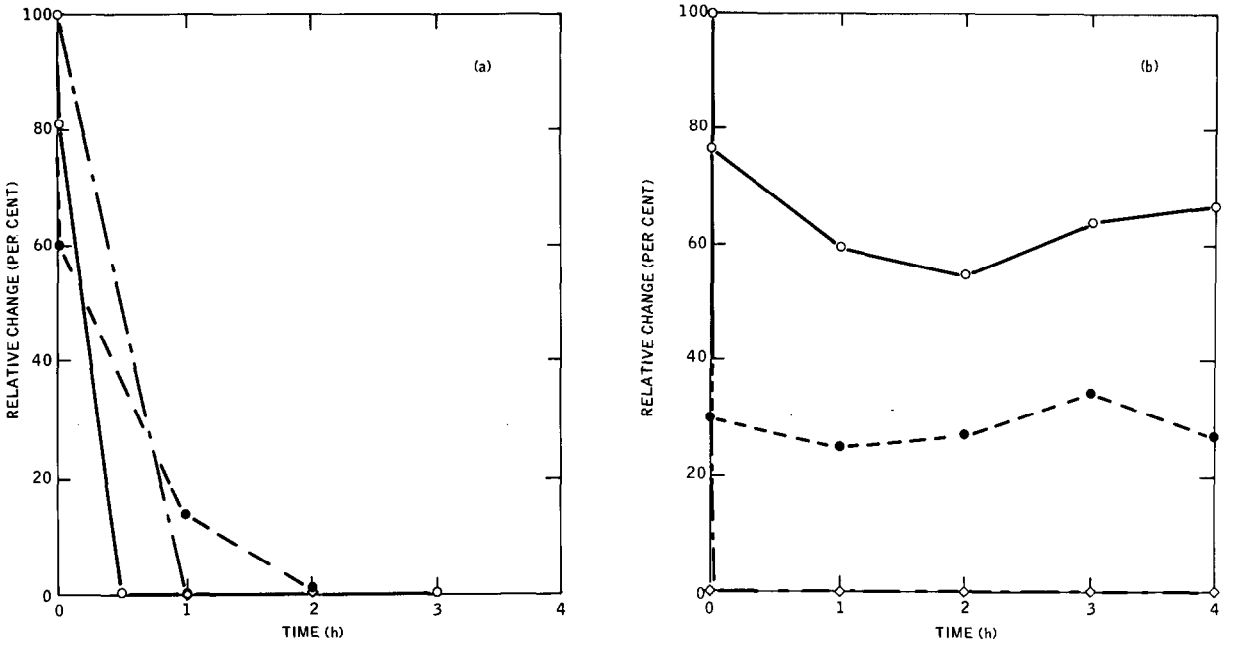

Fig. 1. Effect of DMA (a) and MHI (b) on PCA consumption (○), oxygen consumption (●) and cell viability (◇) at 25°C. Relative changes are based on time zero (no imidoester) as 100% activity and viability. Concentration of the cells and DMA or MHI were 1.5 mg (dry wt)/ml and 5 mg/ml or 10 mg/ml, respectively.

linking with DMA, as opposed to other reagents such as glutaraldehyde, is probably due to the size of the DMA molecule (not exceeding 8.6 Å), and to retention of net charge (protonated amino group to protonated amidine). The structure and chemistry of glutaraldehyde in aqueous solutions are still not resolved [10].

Lower concentrations of the imidoesters produced anticipated results. A decrease in MHI concentration (0.25%) still caused total kill but no change in the PCD activity. A lower concentration of DMA (0.25%) caused total kill but not a complete reduction in activity; 17% of the activity remained after 3 hr at 25°C.

The modification of whole cells, cell components, and purified enzymes with these versatile imidoesters is being pursued.

Acknowledgement

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