MODIFICATION OF THE ESSENTIAL CARBOXYL GROUP IN OCTOPINE DEHYDROGENASE

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1. Introduction

Octopine dehydrogenase (ODH) from muscles of *Pecten maximus* catalyzes the oxidation of D-octopine to L-arginine and pyruvate [1]. This monomeric enzyme of a mol. wt. of 38 000 [2] contains a single –SH and imidazole groups essential for activity [3,4]. The participation of a dicarboxylic amino acid residue in the catalysis seemed possible. Actually, in pig heart NADP-dependent isocitrate dehydrogenase, a single glutamyl residue, situated in the substrate binding site, is essential for the enzymatic reaction [5]. Similarly in dogfish lactate dehydrogenase, aspartate 168 could serve to balance the positive charge of the essential histidine 195 during the oxidoreduction process [6].

Investigation of the role of a –COOH group in ODH was undertaken using water-soluble carbodiimide alone or in the presence of a coloured nucleophile. In the present communication, we describe the results of experiments which demonstrate the loss of catalytic activity after chemical modification of one carboxyl group of the enzyme.

2. Materials and methods

1-Ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride was obtained from Sigma. *N*-(2,4-Dinitrophenyl)ethylenediamine hydrochloride was a product of Calbiochem. Solutions of this amine were made in 2% (v/v) dimethylformamide and the concentrations were determined using DNP group absorbance (Σ^{M}_{360} = 15 000).

ODH was prepared and assayed as previously described [1]. The protein concentration was determined from its absorbance at 280 nm, based on a value of 11.4 for $A^{1\%}_{280}$ [7]. The concentration of the modified enzyme was determined by the biuret method [8].

–SH groups were titrated by DTNB in 8 M urea [9].

Difference spectra were carried out with a Cary 14 spectrophotometer as described in a preceding paper [7].

Abbreviations: Arg + Pyr, arginine + pyruvate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DNP-ethylenediamine, *N*-(2,4-dinitrophenyl)ethylenediamine; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride.

3. Results

3.1. *Inactivation of ODH by EDC*

ODH was inactivated by EDC at concentrations varying from 0.1 to 0.04 M. Fig.1 illustrates the inhibition of ODH by 0.04 M EDC at pH 6 and 25°C. First-order kinetics were observed until 70% inactivation. Under these conditions, the titration of cysteines by DTNB showed that –SH groups were not substituted. Treatment of the inactivated enzyme by 0.75 M NH_2OH at pH 7 for 10 h, did not restore activity, thus indicating that tyrosyl residues were not involved in the reaction with EDC [10].

The protective effect of coenzyme and substrates against inactivation was tested. When NADH was added to the incubation mixture, no appreciable protection

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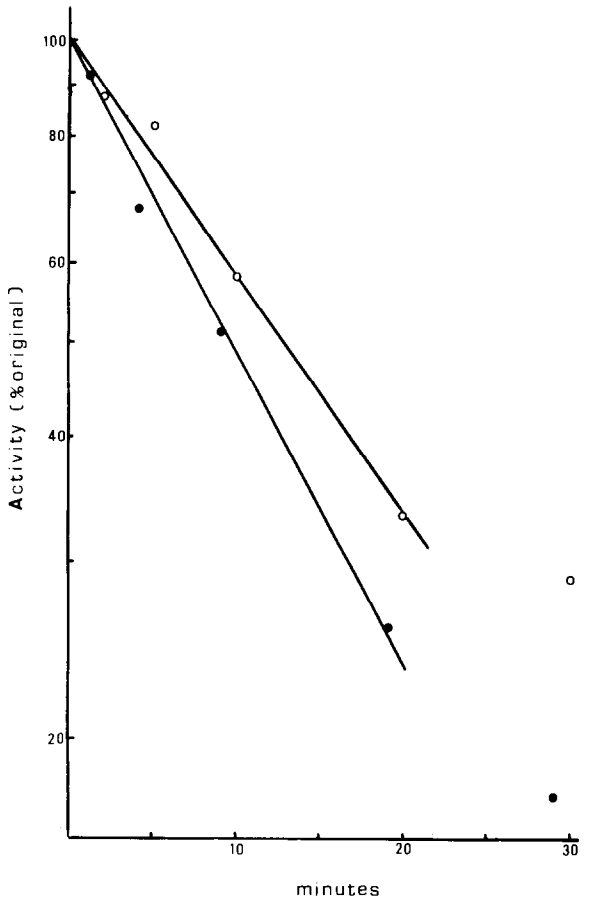

Fig.1. Kinetics of ODH inactivation. 26 μM ODH was reacted in 0.1 M potassium phosphate buffer pH 6 with 0.04 M EDC alone or in the presence of 0.02 M DNP-ethylenediamine. The first-order rate constant k' is 0.05 min^{-1} for EDC alone (○) and 0.07 min^{-1} for EDC + DNP-ethylenediamine (●).

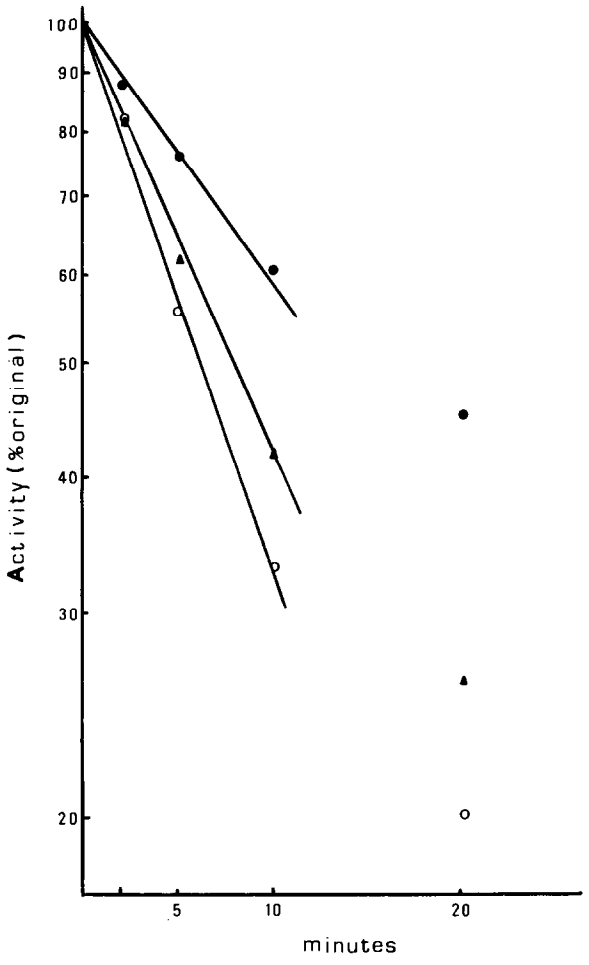

Fig.2. Effect of coenzyme and substrates on the inactivation by EDC. 36 μM ODH was incubated with 0.08 M EDC under experimental conditions described in fig.1. (○) EDC alone. (▲) 0.8 mM NADH added before EDC. (●) 0.8 mM NADH and 0.03 M Arg + 0.03 M Pyr added before EDC.

was observed (fig.2). The same figure shows that a protection of 30% was obtained in the presence of NADH + Arg + Pyr.

The Michaelis constants of coenzyme and substrate were determined for 76% inactivated and native enzymes. The same values, 26 μM for NADH and 1.5 mM for pyruvate, were obtained in both cases. It seems likely that the measurable activity of the 76% inactivated enzyme represents residual unmodified enzyme rather than altered enzyme with distinct kinetic characteristics.

Since it was not possible to assess by kinetic means whether an inactive enzyme is capable of combining with substrates, their ability of binding to enzyme, was studied by using difference spectroscopy [7,11]. Fig.3 (A and B) shows that the 76% inactivated enzyme is still able to bind the coenzyme giving a normal binary complex. Difference spectra resulting from the binding of arginine to native and inactivated holo-enzyme (ODH-NADH) are not similar (fig.3, C and D). The perturbation of the absorption bands of both coenzyme chromophores are missing in the ternary complex with the inhibited enzyme. However, the

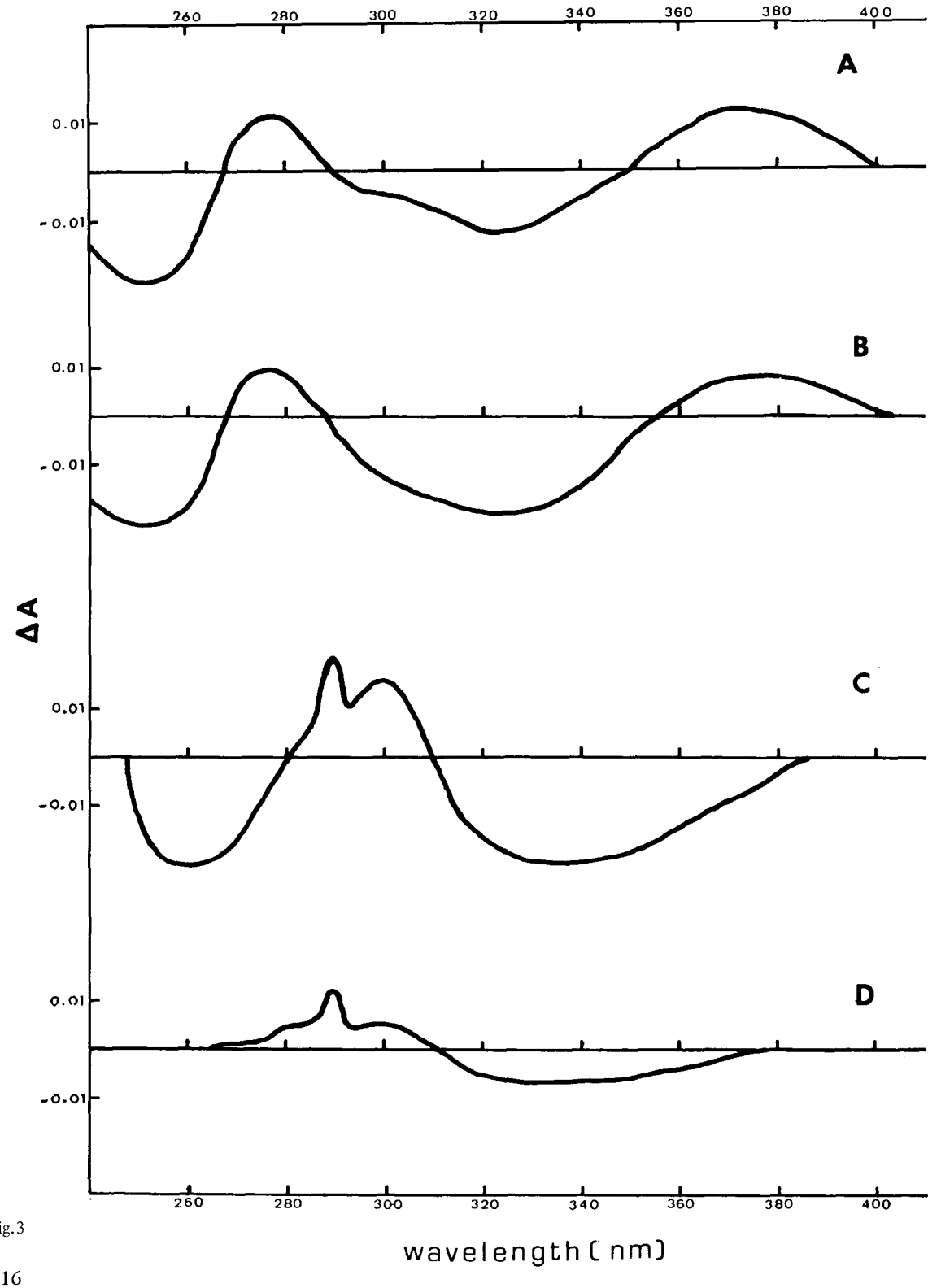

Fig.3

signals corresponding to the perturbation of aromatic residues of the protein are still present.

3.2. *Inactivation of ODH by EDC in the presence of DNP-ethylenediamine*

The DNP-ethylenediamine was used as a nucleophilic reagent to displace the urea derivative from the carbodiimide-carboxyl adduct [12]. The obtained acyl-nucleophile is coloured and this renders possible the evaluation of the number of modified residues.

ODH was incubated with 0.02 M DNP-ethylenediamine in the presence of 0.04 M EDC at pH 6 and 25°C. First-order kinetics of inhibition were observed until 70% inactivation (fig.1). Under these conditions no substitution of –SH groups or other aminoacid residues was observed (as controlled by aminoacid analysis on TSM_1 autoanalyser).

To follow the progress of the incorporation of DNP groups with enzyme inactivation, aliquots of incubation mixture were taken at different times and after passing through a column of Sephadex G-25, the absorbances at 360 nm were measured. As shown in fig.4, the 100% inhibition corresponds to the incorporation of about one mole of DNP per mole of ODH.

The results of protection assays with NADH and substrates are the same as those obtained in the presence of EDC used alone.

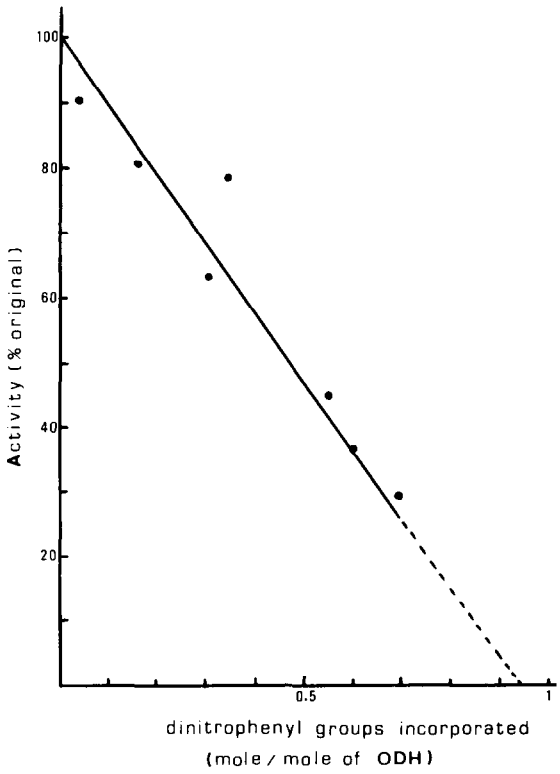

Fig.4. Correlation between inactivation of ODH and incorporation of DNP groups. 15 mg of ODH at a concentration of 1 mg/ml was incubated with 0.02 M DNP-ethylenediamine and 0.04 M EDC in the presence of 0.1 M potassium phosphate buffer pH 6. Samples of 1 mg were taken at different times and passed through 1.4 × 60 cm Sephadex G-25 columns equilibrated with 0.1 M potassium phosphate buffer pH 6 containing 0.1 mM EDTA. For each sample, activity and absorbance at 360 nm were measured.

4. Discussion

The data here presented provide evidence for the involvment of a dicarboxylic amino acid residue in the catalytic function of ODH of *Pecten maximus*. In fact, the inhibition of the enzyme by the carbodiimide alone or in the presence of DNP-ethylenediamine is a specific reaction as can be deduced from aminoacid analysis of histidine, threonine, tyrosine and cysteine contents in the inactivated enzyme. Lysine and threonine modification are unlikely under the experimental conditions of pH and temperature employed [13]. The non-reversibility of the inhibition after treatment by NH_2OH confirms again that the tyrosines are not involved in the reaction with carbodiimide.

The correlation between the extent of inactivation and the incorporation of approximately one mole

Fig.3. Difference spectra of binding of coenzyme and substrate to the native or to the inactivated ODH. 76% inactivated ODH was obtained by treatment with 0.1 M EDC under conditions described in fig.1. The excess of reactants was eliminated by filtration on a column of Sephadex G-25 equilibrated with 0.1 M potassium phosphate buffer pH 6. The concentrations of native and inhibited enzyme were 35 μM. 52 μM NADH was added to the native (A) or to the inactivated (B) ODH. 38 mM Arg was added to the native (C) or to the inhibited (D) holoenzymes (formed with 81 μM NADH).

equivalent of DNP group provides strong evidence that only one carboxyl group is the site of reaction of the carbodiimide and the nucleophile in octopine dehydrogenase.

The unchanged K_Ms of ligands and unmodified differential spectrum of coenzyme binding of strongly inhibited enzyme show that this chemical modification does not affect the conformation of the enzyme.

The pattern of protection afforded by the substrate emphasizes the implication of a carboxyl group in the catalytic process. This group is situated in the active center near the substrate binding site. It seems not to serve directly the fixation of the coenzyme or the substrate but its modification prevents the formation of a catalytically active ternary complex.

The exact nature of the dicarboxylic aminoacid residue essential for the activity of ODH is now under investigation.

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References

[1] Thoai, N. V., Huc, C., Pho, D. B. and Olomucki, A. (1969) Biochim. Biophys. Acta 191, 46–57.
[2] Olomucki, A., Huc, C., Lefébure, F. and Thoai, N. V. (1972) Europ. J. Biochem. 26, 261–268.
[3] Huc, C., Olomucki, A., Pho, D. B. and Thoai, N. V. (1971) Europ. J. Biochem. 21, 161–169.
[4] Thomé-Beau, F. and Olomucki, A. (1973) Europ. J. Biochem. 39, 557–562.
[5] Colman, R. F. (1973) J. Biol. Chem. 248, 8137–8143.
[6] Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossmann, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O. and Taylor, S. S. (1973) Proc. Nat. Acad. Sci. USA 70, 1968–1972.
[7] Pho, D. B., Olomucki, A., Huc, C. and Thoai, N. V. (1970) Biochim. Biophys. Acta 206, 46–53.
[8] Bailey, J. L. (1967) Techniques in protein chemistry pp. 340–352. Elsevier Publishing Company, Amsterdam.
[9] Ellman, G. (1959) Archiv. Biochem. Biophys. 82, 70–77.
[10] Carraway, K. L. and Koshland Jr D. E. (1968) Biochim. Biophys. Acta 160, 272–274.
[11] Olomucki, A., Thomé-Beau, F., Biellmann, J. F. and Branlant, G. (1975) Europ. J. Biochem. 56, 109–116.
[12] Hoare, D. G. and Koshland Jr, D. E. (1967) J. Biol. Chem. 242, 2447–2453.
[13] Kurzer, F. and Douraghi-Zadeh, K. (1967) Chem. Rev. 67, 107–152.