THE REACTION OF *N*-ETHYL MALEIMIDE WITH MALATE DEHYDROGENASE

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1. Introduction

Pig heart mitochondrial malate dehydrogenase (m-MDH) has already been shown to be specifically inactivated by the neutral alkylating reagent, iodoacetamide; the negatively charged reagents, iodoacetate and β-bromopropionate, were found to have no effect on the enzyme's activity [1, 2]. It was also previously established that this inactivation is due to the reaction of iodoacetamide with two reactive histidine residues per molecule of enzyme [1]. Consequently, it was of interest to see if *N*-ethyl maleimide (NEM), also an uncharged molecule, would irreversibly inhibit m-MDH by reaction with these same histidine residues. NEM was in fact found to inactive m-MDH but by reaction with the enzyme's thiol groups and this reaction appears to be non-selective with all or the majority of the thiol groups being blocked before complete loss of activity is achieved. The inhibition pattern of m-MDH with NEM was also more complex than that with iodoacetamide, plots of the logarithm of remaining enzyme activity against time were biphasic below pH 8.2 and linear above this pH.

2. Experimental

m-MDH was the same preparation as used previously and was assayed by the same procedure [1, 2]. NEM was from Sigma, 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was from BDH and both were used as supplied; other materials were the same as before [1, 2]. Assay buffer (0.1 M sodium potassium phosphate pH 7.40) and incubation buffers (sodium potassium phosphate of ionic strength $I = 0.2$) were made up and purified as previously described [1] and the pH of incubation mixtures above pH 7.80 maintained in the pH-stat [2]. All incubations were performed in sodium potassium phosphate solutions $I = 0.2$ and at 25°, enzyme (5 μg/ml), NEM (5 or 20 mM), ligands and buffer were incubated in a total volume of 2 ml: Enzyme stability was checked in control incubations without NEM.

In order to estimate the number of free thiol groups per molecule of m-MDH after inhibition by NEM, a separate inactivation was performed using a larger quantity of m-MDH. 1.16 mg of m-MDH was inactivated at pH 8.20, 25° with 20 mM NEM in a total volume of 2 ml of disodium hydrogen phosphate solution $I = 0.2$. The reaction was followed by assaying for enzyme activity suitably diluted aliquots of the incubation mixture. After 55 min, when 93% of the enzyme activity had been lost, the reaction was stopped by the addition of about 10 μl of 6 N HCl (final pH 6.6) and the mixture was transfered to a test tube in an ice bath. The inactivated m-MDH was dialysed against two changes of 1 litre of 0.1 M phosphate buffer pH 7.40, 2 mM EDTA. The protein concentrations of NEM-inactivated and native m-MDH were estimated by the method of Lowry et al. [3].

DTNB titrations of native and NEM-inactivated m-MDH were performed in split compartment cells and the change in absorbance at 412 nm followed in the Cary 14 recording spectrophotometer. One side of the smaple and reference cells contained 1ml of either native m-MDH (0.108 mg/ml) or 93%-inactivated m-MDH (0.95 mg/ml) in 0.1 M phosphate buffer pH 7.40, 2 mM EDTA. The other compartment of each cell contained 1 ml of 1 mM DTNB in 10 M urea. The urea was dissolved in the required volume of 0.1 M

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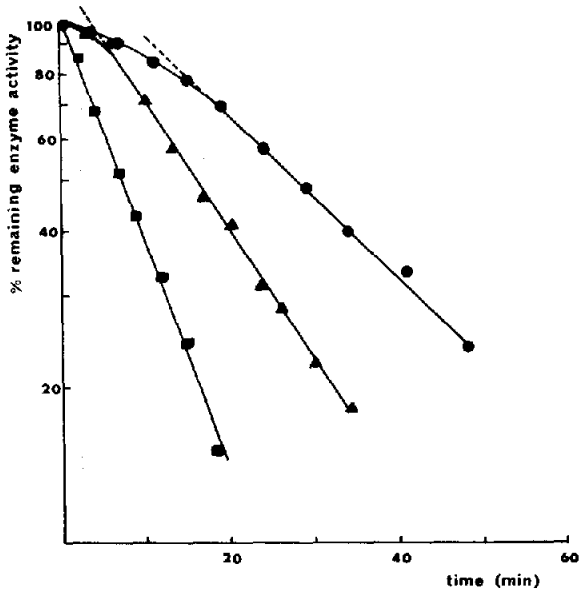

Fig. 1. Progress curves of inactivation of pig heart m-MDH by NEM at different pHs. • = pH 7.80, ▲ = pH 8.20, ■ = pH 8.60. All incubations were in phosphate buffer $I = 0.2$ and with 20 mM NEM at 25°. The logarithm of remaining enzyme activity is plotted against time.

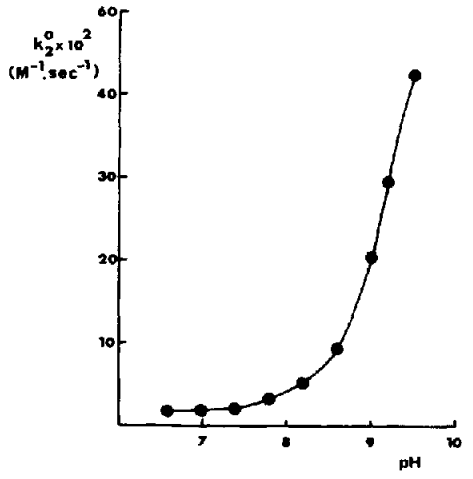

Fig. 2. Inactivation of pig heart m-MDH by NEM as a function of pH. NEM concentration was 5 mM at pH 9 and above and 20 mM below pH 9, incubations were in phosphate buffer $I = 0.2$ at 25°. The observed second order rate constants are plotted against pH.

phosphate buffer pH 7.40 and the pH readjusted to pH 7.40 with HCl. After mixing the sample cell in each experiment, the reaction was allowed to go to completion (about 40 min) and then the reference cell was mixed to check that the base line was restored, this also acted as a duplicate.

3. Results

3.1. *Inactivation experiments*

NEM was found to inactivate m-MDH, but unlike alkylation with iodoacetamide [1,2], the plots of logarithm of remaining activity against time were not linear over the pH range 6.6 to 8.2. Between these pHs the log plots appeared to be biphasic, the rate of inactivation increased until about 30% of the enzyme activity had been lost and then the rate was constant down to zero activity. Above pH 8.2 the log plots appear to be completely linear, but this may be due to the inactivation rate being so rapid that it is not possible to detect an initial slow phase. Fig. 1 shows some typical inactivation profiles at different pHs. It was assumed that the linear portion of these log plots reflected a pseudo first order reaction.

As the slope of the linear portion of the log plots increases with increasing pH, a pH-rate profile of inactivation was plotted with the rate constants obtained from these linear portions (fig. 2). The second order rate constants and not the apparent first order rate constants were plotted as it was necessary to use two different concentrations of NEM (5 and 20 mM). The second order rate constants were computed from the apparent first order rate constants by dividing each by the particular concentration of NEM used.

As can be seen from fig. 2, the pH profile shows continually increasing reactivity of m-MDH towards NEM with increasing pH. It would appear that NEM does not react with the histidine residue alkylated by iodoacetamide as the pH-rate profile would then be expected to reflect a pK_a of 7.1 for the reacting group(s) [2].

As there is a low and constant rate of inactivation at neutral pHs and the rate of inactivation increases rapidly above pH 8, it was suspected that the NEM was reacting with the thiol groups of m-MDH. The free thiol contents of native and NEM-inactivated m-MDH were therfore estimated by DTNB titration.

Table 1

Estimation of the number of free thiol residues per molecule of native and NEM-inactivated m-MDH. The figures are the mean of those obtained on mixing firstly the sample cell and then the reference cell.

Experiment	Change in absorbance at 412 nm	Concentration of free thiols (μM)	Concentration of of protein after mixing split compartment cells (μM)	Number of free thiols/molecule of m-MDH
Native m-MDH	0.1358	10.5	0.77	14
93%-inactivated m-MDH	0.0236	1.74	0.68	2.6

Using a molar extinction coefficient for the mercaptide ion of 13,600 at 412 nm [4] and assuming a molecular weight of 70,000 for m-MDH [5,6], the number of free thiol groups per molecule of native and 93%-inactivated m-MDH were found to be 14 and 2.6 respectively. The results of the DTNB titration are presented in table 1. The estimate of 14 free thiols per molecule of native enzyme is in good agreement with previously reported figures for the cysteine content of m-MDH [1, 7, 8].

3.2. *Coenzyme protection*

NAD (10 mM) and NADH (1 mM) only partially prevented inactivation of m-MDH by NEM (20 mM) at pH 7.80. Plots of the logarithm of remaining activity against time were linear and not biphasic. The rate of inactivation in the presence of either coenzyme was slower than the linear phase of inactivation in their absence at the same pH; NAD reduced the rate by 2.4 fold and NADH by 4.3 fold. The concentrations of coenzymes in these experiments were virtually saturating as the dissociation constants of the binary complexes of m-MDH with NAD and NADH under identical con ditions are 400 μM and 10 μM respectively [1].

4. Discussion

As the reaction of NEM with a histidine residue would be an addition reaction whereas the reaction of iodoacetamide with m-MDH is a nucleophilic substitution, it is not possible to interpret in terms of the enzyme's properties the absence of reaction of the reactive histidine residue of m-MDH with NEM.

As as many as 11–12 of the 14 thiol groups of m-MDH reacted per molecule to produce 93% inactivation, it would seem that inactivation by NEM is via a non-selective reaction with the enzyme thiol groups. If m-MDH posses any "essential" thiol groups then they are certainly not especially reactive towards alkylating reagents and the NEM data, in fact, suggests that none are involved in catalysis as for complete inactivation all or most of them must be blocked. These results therefore seem to confirm those from previous studies on m-MDH with the thiol specific reagent *p*-chloromercuribenzoate (PCMB), it was found that for complete loss of activity all the thiol specific blocked by PCMB [5, 7, 9]. Presumably and not surprisingly, reaction with all of the thiols by either PCMB or NEM produces a loss of structural integrity.

There is also a precedent for the biphasic inhibition pattern observed with NEM below pH 8.2. m-MDH from ox kidney has been reported to behave similarly with DTNB, the initial reaction with DTNB is slow but increases as more thiols titrate [10]. It was assumed that reaction with DTNB of the first one or two thiols caused an opening up of the molecule and thereby exposing the remaining residues. The same could be happening with pig heart m-MDH on reaction with NEM.

Although NEM reacts with all or most of the cysteine residues of m-MDH, it is not altogether surprising that the coenzymes can reduce the rate of reaction. It has already been reported that the substrates and coenzymes can reduce, but not prevent, the reaction of DTNB with pig heart m-MDH, even in 7.2 M urea [11].

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