MALEYLACETONE *CIS–TRANS* ISOMERASE: FORMATION OF AN *N*-ETHYLMALEIMIDE-LABELED ENZYME ONLY DURING THE SLOW PHASE OF THE BIPHASIC INHIBITION REACTION

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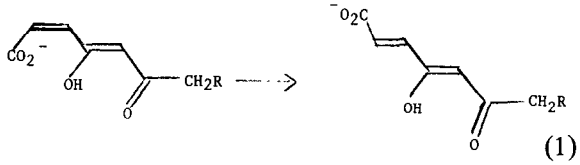
1. Introduction

Maleylacetone *cis–trans* isomerase from *Vibrio* 01 catalyzes the reaction shown in eq. (1) (R = H or CO_2^-) [1]. Glutathione is required as a coenzyme.



(1)

Previous reports from this laboratory have discussed the mechanism of GSH-interaction with the substrate in the enzymatic reaction [2,3]. The enzyme which is highly labile to air oxidation, can be protected with mercaptoethanol and/or EDTA. The isomerase has been reported to be irreversibly inactivated by NEM and hence believed to have an important thiol group [1]. Here, we show that the kinetics of NEM-inactivation is biphasic. Covalent attachment of the inhibitor to the only cysteinyl thiol occurs in the slower phase. No covalent attachment of NEM in the rapid phase can be detected.

2. Materials and methods

GSH, M_r marker proteins, and SDS were purchased from the Sigma Chemical Co. Mercaptoethanol and NEM were obtained from the Aldrich Chemical Co. All salts were reagent grade. [2,3-^{14}C]NEM (8–10 mCi/mmol) was purchased from Amersham. Argon (high purity, 99.998%) was obtained from Matheson. *N*-Ethylsuccinimide was obtained by treatment of ethyl iodide with succinimide in $CH_3OH/NaOCH_3$. The product was distilled. NMR ($CDCl_3$): δ 2.80 (*S*,2 H); 1.18 (*t*,3 H); 3.65 (*q*,2 H).

The enzyme was purified up to and including the GS–agarose affinity chromatography step in [2]. Further purification was accomplished by a Sephadex G-75 followed by a DEAE-cellulose treatment. The purity of the enzyme was examined by slab (1.2 mm) SDS–polyacrylamide gel electrophoresis. Prior to application, the enzyme was treated as in [4]. M_r marker proteins were run alongside the enzyme. The enzyme was stored in 0.1% mercaptoethanol solution containing 10 mM EDTA and 10 mM phosphate buffer (pH 7.3) at 2°C. Prior to NEM-treatment mercaptoethanol was removed by repeated dialysis against argon-purged buffer solutions containing EDTA. Amino acid analysis of the 6 N HCl-hydrolyzed native enzyme was carried out in the standard way on an amino acid analyzer. The procedure in [5], using performic acid followed by HBr treatment, was used to determine half-cystine residues in the homogeneous native enzyme. Tryptophan was estimated from the A_{280} of the enzyme at neutral pH.

The radiochemical purity of [^{14}C]NEM was examine alongside authentic NEM, by TLC on silica gel using benzene, methanol, acetic acid (4:1:1) as solvent. A single radioactivity spot, having the same R_F as authentic NEM, was found. The kinetics of the reaction of NEM with enzyme were studied under pseudo first-order conditions: [NEM] ≫ [isomerase].

Abbreviations: GSH, glutathione; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; M_r, relative molecular mass

After mixing, aliquots were withdrawn at specified times, added to the assay mixture containing an excess of GSH, and assayed for enzymatic activity. Similar studies were carried out with [^{14}C]NEM. At specified times, aliquots of the isomerase–[^{14}C]NEM solution were removed and added to buffer containing an excess of mercaptoethanol. A portion of the quenched aliquot was assayed for residual enzymatic activity and the remainder subjected twice to gel filtration on either a P-2 (BioRad) or an HPLC I-125 (Waters Associates) column to remove excess [^{14}C]-NEM. The purified enzyme was subjected to 6 N HCl hydrolysis. The hydrolysate was chromatographed and analyzed on an amino acid analyzer. Fractions were collected and counted in a Beckmann Liquid Scintillation Counter.

3. Results

SDS–polyacrylamide gel electrophoresis of the enzyme by the method in [4] produced a single band (fig.1). Comparison of its R_F with those of known proteins (egg albumin, cytochrome *c*, lysozyme, myoglobin, bovine serum albumin) places the M_r of this unit at 16 000. Gel filtration of the isomerase together with marker proteins on an I-125 column suggests that the M_r of the native enzyme is 35 000 ± 3000. The results of amino acid analysis are shown in table 1. One cysteine/M_r 16 000 is found.

The kinetics of inhibition of enzymic activity by NEM is biphasic. In experiments with the pure native enzyme and NEM (1×10^{-4} M) loss of activity follows pseudo first-order kinetics with an average bimolecular rate constant of 5×10^3 $M^{-1} \cdot min^{-1}$ until ~75% of the activity is lost. There the inhibition reaction is essentially stopped at this NEM level. Further inhibition is achieved at higher NEM level. At 4×10^{-2} M NEM, the remaining 25% of activity is lost in a pseudo first-order process with an average bimolecular rate constant of 0.5 $M^{-1} \cdot min^{-1}$ at 0°C. ^{14}C-Labeling of the enzyme was studied at low (2×10^{-5} M, 8.4 mCi/mmol) and high (1×10^{-2} M,

Table 1
Amino acid composition of maleylacetone *cis–trans* isomerase

	Residues/M_r 16 000	
Cys	1.1	1
Asx	12.8	13
Met	1.2	1
Thr	6.4	6
Ser	32.3	32
Glx	26.9	27
Pro	5.6	6
Gly	26.8	27
Ala	13.3	13
Val	5.8	6
Ileu	4.4	4
Leu	6.9	7
Tyr	2.9	3
Phe	3.9	4
Lys	4.7	5
His	3.4	3
Arg	1.0	1
Trp		2

Fig.1. SDS–polyacrylamide slab gel electrophoresis (flow from left to right) of the homogeneous isomerase heated at 100°C for 10 min in a solution containing 1% SDS, 8 M urea and 0.1% dithiothreitol before application of the sample [4].

10 mCi/mmol) levels of [2,3-^{14}C]NEM corresponding to the fast and the slow phases of the inhibition kinetics.

No bound ^{14}C activity can be detected during the rapid loss of 75% of the enzymatic activity. This result was obtained by examining aliquots at various extents of inhibition during the rapid phase [^{14}C]-NEM–enzyme reaction. The enzyme in each aliquot was purified, hydrolyzed, chromatographed, and its constituent amino acids showed no radioactivity. In the slower phase of inhibition, however, the only cysteine present, reacts with NEM as judged by the appearance of ^{14}C activity in an eluted peak just before the appearance of aspartic acid. Corresponding hydrolysis and amino acid analysis of the authentic cysteine–NEM adduct identifies the ^{14}C activity peak as belonging to *S*-(1,2-dicarboxyethyl)-L-cysteine [6]. The time course of the loss of enzymatic activity with concomitant ^{14}C-labeling during incubation of the enzyme with 1×10^{-2} M [^{14}C]NEM is shown in fig.2. The enzymatic activity curve, in the slow phase, extrapolated to zero time, intersects the ordinate at 26% which is the residual activity after completion of the fast phase. If the slow phase is followed by plotting that fraction of the 26% remaining activity *vs* the nmol cysteine labeled with [^{14}C]NEM/nmol subunit (M_r 16 000), a linear relationship is obtained (fig.3).

Variations of the above experiment were carried out. In one, enzyme was inhibited with $\sim 1 \times 10^{-4}$ M

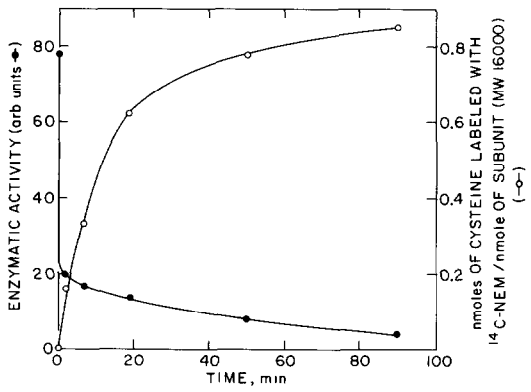


Fig.2. Enzymatic activity and no. cysteine residues/enzyme molecule labeled as a function of time during reaction of enzyme with 1×10^{-2} M [^{14}C]NEM (10.0 μCi/μmol) at pH 7.4. EDTA was present but extraneous thiol compounds were absent.

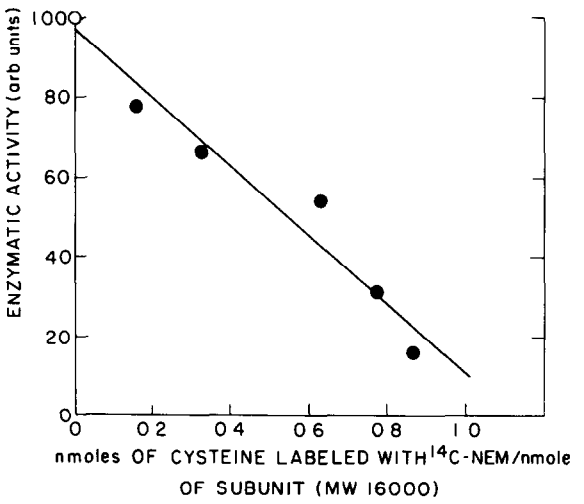


Fig.3. Enzymatic activity *vs* no. cysteine residues/enzyme molecule labeled with [^{14}C]NEM in the slow phase of reaction.

[^{14}C]NEM. The enzyme was reisolated, purified by gel filtration and not hydrolyzed but counted directly. No ^{14}C incorporation could be detected. In another experiment, the enzyme was incubated with unlabeled NEM ($\sim 10^{-4}$ M) whereupon the usual 75% activity was lost. Dialysis against mercaptoethanol and EDTA did not restore activity. After removal of mercaptoethanol the enzyme was incubated with [^{14}C]NEM (4×10^{-2} M) after which all enzymatic activity was lost and the enzyme was found to be radioactively labeled.

The effect of *N*-ethylsuccinimide on the rate of enzymatic reaction was examined. Incubation of the enzyme with 1.3×10^{-3}–1.3×10^{-2} M *N*-ethylsuccinimide for 10 min prior to assay did not reduce the enzymatic activity perceptibly.

4. Discussion

Maleylacetone *cis–trans* isomerase from *Vibrio* 01 has been thought to have an important thiol group since it was known that it is highly labile in the absence of mercaptoethanol or EDTA and that it is irreversibly inhibited by NEM [1]. Indeed, amino acid analysis here shows that 1 cysteine residue is present/subunit M_r 16 000. No other half-cysteines are present. The kinetics of inhibition by NEM, however, are atypical. There is an extremely fast pseudo first-order reaction leading to loss of 75% of the enzymatic

activity. At a higher concentration of NEM the remaining 25% of activity is lost in another pseudo first-order reaction. The first phase proceeds 10^4-times faster than the second one. In order to identify the group(s) on the enzyme which are altered during NEM inhibition, incubations with appropriate concentrations of [^{14}C]NEM in the two phases were carried out. Enzyme isolated from reaction during the fast phase contains no ^{14}C-label. During the slow phase of inhibition, however, the only cysteine thiol adds irreversibly to NEM and thereby the enzymatic activity is reduced to zero. Loss of residual enzymatic activity in this slow phase shows a 1:1 correlation with cysteine thiol alteration. The nature of the reaction of the enzyme with NEM during the fast phase is obscure at present.

To test whether NEM might be reacting with an uncommon residue which perhaps might not be an amino acid, the enzyme after inhibition by [^{14}C]NEM in the fast phase was purified and counted intact. Here, too, the enzyme was found not to be labeled. Moreover, even if the enzyme is not labeled it is irreversibly inhibited during the fast phase, since activity can not be restored with mercaptoethanol.

To test whether NEM might be promoting an inhibitory conformational change, the enzyme was incubated with an analogue, *N*-ethylsuccinimide. At a 100-fold higher concentration than used for NEM inhibition no effect on enzymatic activity could be seen. While this experiment does not itself rule out a conformational change this explanation appears untenable because of the structural similarities of the two imides.

Thus these results show that there are two separate NEM-inhibitory processes. The slower is of the type generally observed for thiol enzymes. The ability of NEM to inhibit isomerase activity irreversibly in a 10^4-fold faster reaction and to do so by a mechanism which does not lead to covalent binding of the inhibitor to the protein, is unique. The elucidation of the mechanism of this rapid inhibition reaction must await further study.

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