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Inhibition of *Saccharomyces cerevisiae* Phosphomannose Isomerase by the NO-donor S-nitroso-acetyl-penicillamine

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Phosphomannose isomerase (PMI; EC. 5.3.1.8) is an essential metalloenzyme in the early steps of the protein glycosylation pathway in both prokaryotes and eukaryotes. The Cys150 residue (according to *Candida albicans* PMI numbering) is conserved in the active centre of mammalian and yeast PMI, but not in bacterial species where it is replaced by Asn. Here, the dose- and time-dependent inhibitory effect of the NO-donor S-nitroso-acetyl-penicillamine on the *Saccharomyces cerevisiae* PMI catalytic activity is reported. The analysis of the X-ray crystal structure of *C. albicans* PMI and of the molecular model of *S. cerevisiae* PMI provides a rationale for the low reactivity of Cys150 towards alkylating and nitrosylating agents.

Keywords: Phosphomannose isomerase, S-nitroso-acetyl-penicillamine, Nitric oxide, Enzyme inhibition, Yeast, *Saccharomyces cerevisiae*

Abbreviations: PMI, phosphomannose isomerase; SNAP, S-nitroso-acetyl-penicillamine; NAP, N-acetyl-penicillamine

INTRODUCTION

Phosphomannose isomerase (PMI; EC. 5.3.1.8) is an essential enzyme in the early steps of the protein glycosylation pathway in both prokaryotes and eukaryotes. Lack of the enzyme is lethal for fungal organisms and it is thus a potential fungicidal target. PMI catalyses the reversible isomerization of fructose 6-phosphate and mannose 6-phosphate. This is the first step in the synthesis of cell wall mannosylated glycoproteins and other N- and O-linked oligosaccharides from glycolytic intermediates. Therefore, PMI plays a central role in yeast cell wall biosynthesis.[1,2]

PMI is a metalloenzyme containing one tetracoordinated zinc ion which is essential for catalysis.[3] Cations, such as zinc and cadmium,

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competitively inhibit *Candida albicans* PMI with respect to the substrate mannose-6-phosphate.[4] *C. albicans* PMI has been shown to be inhibited also by mercury and silver ions in a time-dependent and irreversible fashion.[5,6] Notably, the binding site of mercury and silver ions has been shown to be distinct from that of zinc and cadmium, and to involve the Cys150 residue, located 9 Å away from the catalytic metal.[5–7]

The Cys150 residue (according to *C. albicans* PMI numbering)[7] has been also identified in the active centre of PMI from *Saccharomyces cerevisiae*.[4,7,8] This cysteine residue is conserved in mammalian and yeast PMI, but not in bacterial species where it is replaced by Asn.[8] According to cysteine reactivity properties, the Cys150 residue of PMI from *C. albicans* undergoes oxidation by iodoacetate, with the concomitant loss of enzyme activity.[8]

Here, the dose- and time-dependent inhibitory effect of the NO-donor S-nitroso-acetyl-penicillamine (SNAP) on the *S. cerevisiae* PMI catalytic activity is reported. Notably, the NO-donor SNAP modulates the function of Cys-containing proteins (e.g., cysteine proteases) via S-nitrosylation and mixed disulphide formation (see Figure 1).[9,10]

MATERIALS AND METHODS

S. cerevisiae phosphomannose isomerase (PMI; EC. 5.3.1.8), *S. cerevisiae* glucose-6-phosphate dehydrogenase, *S. cerevisiae* phosphoglucose isomerase, mannose-6-phosphate sodium salt, $NADP^+$, S-nitroso-acetyl-penicillamine (SNAP; see Figure 1), and N-acetyl-penicillamine (NAP; see Figure 1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals

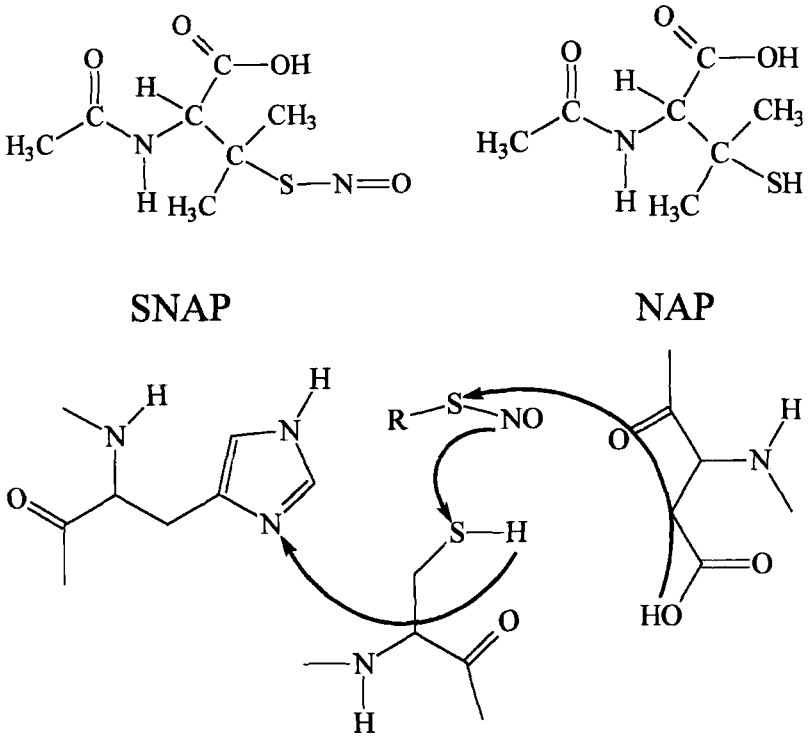

FIGURE 1 Chemical structures of the NO-donor S-nitroso-acetyl-penicillamine (SNAP) and of its de-nitrosylated derivative N-acetyl-penicillamine (NAP) (top panel). Schematic representation of the Cys *trans*-nitrosylation reaction catalytically assisted by neighbouring His and Asp residues acting as base and acid, respectively. R–S–NO indicates a nitrosothiol reagent (bottom panel). Note that other residues (e.g., Glu, Tyr, Lys and Arg) can act as acid and/or base in the reaction.[9,17]

were from Merck AG (Darmstadt, Germany). All products were of analytical or reagent grade and were used without further purification.

The catalytic activity of *S. cerevisiae* PMI was measured at 37 °C using a coupled assay in which the product fructose-6-phosphate was converted to glucose-6-phosphate. This was then oxidised to 6-phosphogluconate, and the concomitant reduction of the $NADP^+$ was monitored by absorbance changes at 340 nm. Assays were carried out in a microtitre plate, in a total volume of 500 µL, containing 1.0×10^{-1} M Tris-HCl buffer (pH = 8.0), 45 units of *S. cerevisiae* PMI, 45 units of *S. cerevisiae* glucose-6-phosphate dehydrogenase, and 12.5 units of *S. cerevisiae* phosphoglucose isomerase per assay.[4,8]

The effect of SNAP and NAP on the catalytic activity of *S. cerevisiae* PMI was determined, at pH 8.0 (1.0×10^{-1} M Tris-HCl) and 25 °C, by incubation of the enzyme with the inhibitor. The *S. cerevisiae* PMI/SNAP or PMI/NAP incubation time ranged between 30 min and 3 h. SNAP and NAP concentrations ranged between 1.0×10^{-3} M and 1.0×10^{-2} M. Then, *S. cerevisiae* PMI/SNAP or PMI/NAP solution was added to the reaction mixture (containing *S. cerevisiae* glucose-6-phosphate dehydrogenase, yeast *S. cerevisiae* phosphoglucose isomerase, mannose-6-phosphate, $NADP^+$, and $MgCl_2$) and the catalytic activity assayed.[4,8]

SNAP and NAP did not affect *S. cerevisiae* glucose-6-phosphate dehydrogenase and *S. cerevisiae* phosphoglucose isomerase activity. In fact, *S. cerevisiae* PMI activity[4,8] was unaffected by pre-incubation of *S. cerevisiae* glucose-6-phosphate dehydrogenase and *S. cerevisiae* phosphoglucose isomerase with SNAP or NAP. The pre-incubation time ranged between 30 min and 3 h, and the SNAP and NAP concentration ranged between 1.0×10^{-3} M and 1.0×10^{-2} M.

Under all the experimental conditions, the absorbance change was linear over the assay time (2 min). The slope of absorbance over time for each inhibitor concentration was compared with that of controls in multiple assays. In detail, experiments were carried out in triplicate, the S.D. being lower than ±7%.

Values of the apparent pseudo first order rate constant (i.e., k) for PMI inhibition by SNAP were determined from the time-dependence of enzyme inactivation at fixed SNAP concentration (i.e., [SNAP]) under conditions where [SNAP] ≫ [PMI] (see Figure 2), according to Equation (1).[10]

$$[PMI^*] = [PMI] \times (1 - e^{-kt}), \qquad (1)$$

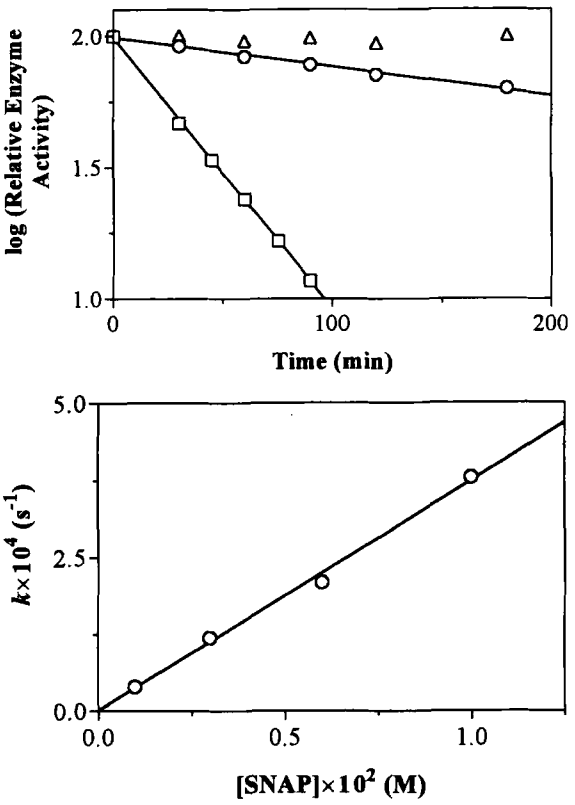

FIGURE 2 Time course of *S. cerevisiae* PMI inactivation by SNAP (top panel). The enzyme activity was assayed after treatment with 1.0×10^{-3} M (○) and 1.0×10^{-2} M (□) SNAP. The effect of 1.0×10^{-2} M NAP (△) on the *S. cerevisiae* PMI activity is shown for comparison. The continuous lines were calculated according to Equation (1) with the following values of the pseudo first order rate constant (i.e., k) for PMI inhibition by SNAP: $3.8 \times 10^{-4}\,s^{-1}$ (□) and $4.0 \times 10^{-5}\,s^{-1}$ (△). Dependence of the pseudo first order rate constant k on SNAP concentration (bottom panel). The continuous line was calculated according to Equation (2) with $k_2 = 3.7 \times 10^{-2}\,M^{-1}s^{-1}$. Data were obtained at pH 8.0 and 25 °C. For further details, see text.

where [PMI*] is the inhibited enzyme concentration and [PMI] is the total active enzyme concentration at $t = 0$. The value of the second order rate constant for PMI inhibition by SNAP (i.e., k_2) was determined from the linear dependence of k on [SNAP] (see Figure 2), according to Equation (2).[10]

$$k = k_2 \times [\text{SNAP}]. \quad (2)$$

The three-dimensional structure of *S. cerevisiae* PMI was modelled using the X-ray crystal structure of *C. albicans* PMI (PDB code: 1PMI)[7] as a template. In detail, sequence alignment was obtained using the program BLAST[11] (available at www.ncbi.nlm.nih.gov/BLAST). Secondary structure prediction was carried out using PredictProtein[12] (available at dodo.bioc.columbia. edu/predictprotein/). Finally, the three-dimensional structure of *S. cerevisiae* PMI was built using Modeller (Release 4),[13] a program that models protein three-dimensional structure by satisfaction of spatial restraints. Solvent accessibility of cysteine residues was calculated in *C. albicans* PMI, *S. cerevisiae* PMI, and papain (PDB code: 1BQI)[14] using the program SURFCV.[15] Graphic representation of the 3D structure of *S. cerevisiae* PMI was made with the program GRASP.[16]

RESULTS AND DISCUSSION

The NO-mediated chemical modification of (macro)molecular targets, such as Cys-containing proteins, occurs via (trans)nitrosylation reactions mediated by NO-donors, often facilitated by neighbouring residues acting as base and acid (see Figure 1).[9,17] In particular, the great propensity for nitrosothiol formation represents a modulation mechanism of (macro)molecules containing NO-reactive Cys residues at their active centre(s) and/or allosteric site(s). Notably, cysteine proteases (e.g., papain) are considered as paradigms for NO-mediated S-nitrosylation and mixed disulfide formation.[9,10,17–21]

Figure 2 shows that *S. cerevisiae* PMI is inactivated dose- and time-dependently by the NO-donor SNAP. Conversely, the NO-deprived compound NAP does not affect *S. cerevisiae* PMI activity (see Figure 2). Furthermore, in agreement with the results obtained for *C. albicans* PMI,[8] iodoacetate inactivates *S. cerevisiae* PMI (data not shown).

The reactivity of Cys150 residue towards alkylating agents as well as mercury and silver ions[4–6,8] suggests that this residue likely represents the SNAP-target in *S. cerevisiae* PMI. In this regard, it is worthwhile mentioning that molecular modelling of *S. cerevisiae* PMI shows that out of the five Cys residues present in the protein, Cys150 is the only solvent accessible one, though its accessibility is restricted to part of the sulphydryl group. A similar situation is observed in *C. albicans* PMI, in which five Cys residues are present and only the sulphydryl groups of Cys150 and Cys261 are partially exposed to solvent.

The value of the second order rate constant for the SNAP-induced inactivation of *S. cerevisiae* PMI ($= 3.7 \times 10^{-2}$ $M^{-1} s^{-1}$, at pH 8.0 and 25 °C; present study) is about 400-fold lower than that reported for papain inhibition ($= 15\, M^{-1} s^{-1}$, at pH 6.5 and 25 °C).[10] Again, the value of the second order rate constant for the iodoacetate-induced inactivation of *C. albicans* PMI ($= 7.0 \times 10^{-2}\, M^{-1} s^{-1}$, at pH 9.0 and 25 °C)[8] is lower than that reported for papain inhibition ($= 1.1 \times 10^{3}\, M^{-1} s^{-1}$, at pH 5.5 and 25 °C, and $20\, M^{-1} s^{-1}$, at pH 10.1 and 25 °C).[22] These data reflect the lower reactivity of the Cys150 residue present at the yeast PMI active centre with respect to that of the Cys catalytic residue of cysteine proteases (e.g., Cys25 of papain).[8,9]

The analysis of the X-ray crystal structure of *C. albicans* PMI and of the molecular model of *S. cerevisiae* PMI provides a rationale for the low reactivity of Cys150 in these two enzymes towards alkylating and nitrosylating agents. In detail, the solvent accessibility of the Sγ atom of the Cys150 residue of *C. albicans* and *S. cerevisiae*

PMI is much lower than that of the catalytic Cys25 Sγ atom of papain (0.15 Å^2, 1.5 Å^2, and 14 Å^2, respectively). Moreover, the presence of the carboxyl group of Asp283 at only 4 Å from Cys150 Sγ atom (see Figure 3) and the rather apolar environment surrounding Cys150 are expected to stabilize the protonated form of the Cys sulphydryl group, which is known to be poorly reactive towards NO and NO-donors (e.g., SNAP).[9,17] Conversely, the papain Cys25 residue is hydrogen bonded to His159 which stabilizes the dissociated form of the Cys25 sulphydryl group thus facilitating nitrosylation and/or mixed disulphide formation.[9,10]

As already observed, although Cys150 lies at the bottom of the active site cleft of yeast PMI, it is approximately 9 Å away from the catalytic zinc ion. Thus, it is unlikely that chemical modification of the Cys150 Sγ atom can cause direct steric hindrance to substrate binding. More likely, chemical modification of Cys150 causes local change(s) in the protein conformation, leading to an alteration of the active site geometry.[7] Notably, the Cys150 Sγ atom is located only 3.7 Å away from His285, one of the residues coordinating the zinc ion (see Figure 3). Thus it is likely that *S*-nitrosylation of Cys150 may lead to a perturbation of the His285 geometry, affecting enzyme catalysis.

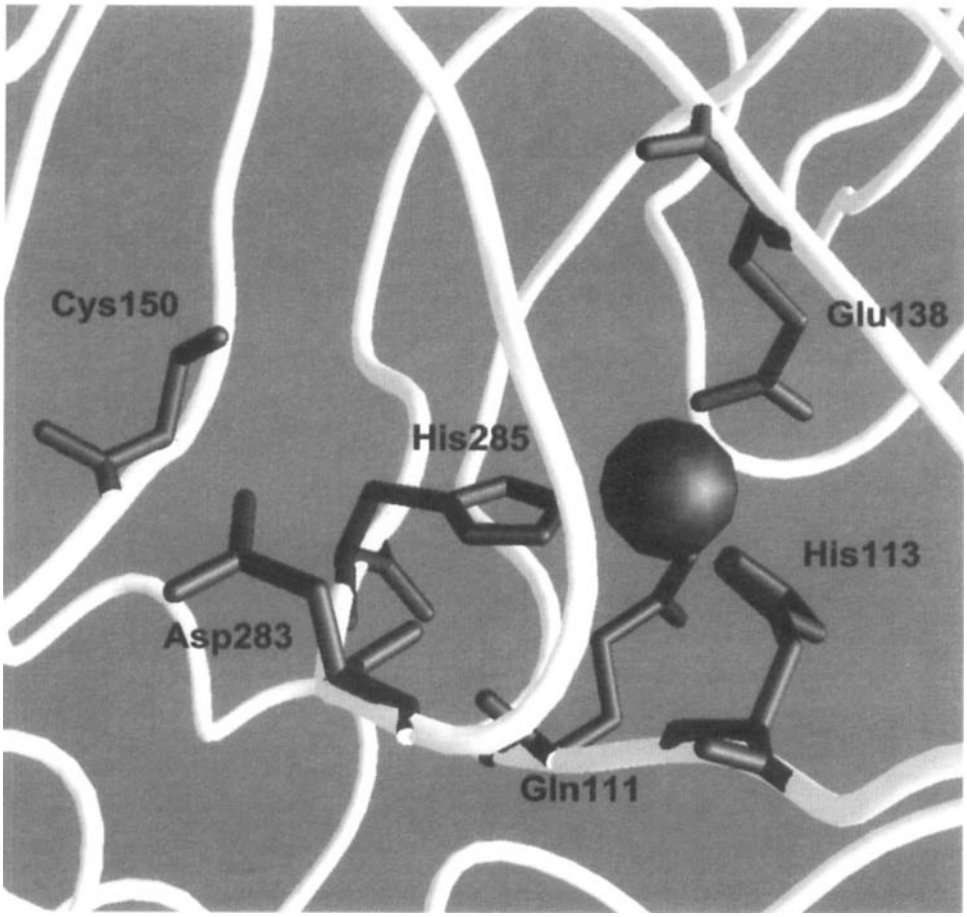

FIGURE 3 Schematic representation of the zinc binding site of *S. cerevisiae* PMI. The zinc ion is represented by a black sphere. For sake of clarity only the zinc ligands (Gln111, His113, Glu138, and His285), Cys150, and Asp283 are shown. Residues are numbered according to *C. albicans* PMI.[7] The figure was made with the program GRASP.[16] For further details, see text.

As a whole, the present data confirm that the reactivity of the cysteine sulphydryl groups towards nitrosylating agents is affected by electrostatic and chemical characteristics of the local environment. In particular, solvent accessibility, environment polarity and charge distribution appear to be key factors modulating the susceptibility of Cys residues to NO-mediated chemical modification.[9]

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