Interaction of rhodanese with intermediates of oxygen reduction

Carlo Cannella and Rodolfo Berni, with the technical assistance of Palmerino D'Ottavio+

Institutes of Biochemistry and Molecular Biology, University of Parma, Via del Taglio, 43100 Parma and *+Institute of Biological Chemistry, 'La Sapienza' University of Rome, P.le A. Moro 5, 00185 Rome, Italy*

Received 1 August 1983

Cyanide-promoted inactivation of the enzyme rhodanese [thiosulfate sulfurtransferase (EC 2.8.1.1)] in the presence of ketoaldehydes is caused by reduced forms of molecular oxygen generated during autoxidation of the reaction products. The requirement of both catalase and superoxide dismutase to prevent rhodanese inactivation indicates that hydroxyl radical could be the most efficient inactivating agent. Rhodanese, also in the less stable sulfur-free form, shows a different sensitivity towards oxygen activated species. While the enzyme is unaffected by superoxide radical, it is rapidly inactivated by hydrogen peroxide. The extent of inactivation depends on the molar ratio between sulfur-free enzyme and oxidizing agent. Fully inactive enzyme is reactivated by reduction with its substrate thiosulfate.

Rhodanese *Phenylglyoxal* *Cyanide* *Thiosulfate* *Superoxide radical* *Hydrogen peroxide*

1. INTRODUCTION

A novel reaction of ketoaldehydes in the cyanide-dependent promotion of disulfide bond formation in the active site of rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) was reported in [1]: after removal of persulfide sulfur from the active site of sulfur-rhodanese (E-S-SH), phenylglyoxal (PGO) behaves as powerful inactivating agent towards the resulting sulfur-free enzyme (E-SH) only in the presence of a small excess of cyanide. Although PGO is specific for chemical modification of arginine residues [2], the cyanide-dependent inactivation caused by this reagent consists in the formation of an intramolecular disulfide bond between the essential and neighboring sulfhydryl groups [1]. The role of cyanide in transforming PGO into a powerful oxidizing agent for sulfhydryl groups was regarded as a mystery [1,3]. A similar inactivation promoted by low concentration of dithiothreitol (DTT) is caused by hydrogen peroxide generated during DTT autoxidation [4]. Since the chemical modification in the enzyme inactivated by PGO and cyanide consists in the oxidation of the essential sulfhydryl group, it was of interest to investigate the role of oxygen in the course of this reaction. Here, we show that intermediates of oxygen reduction are produced by reaction of cyanide with PGO or during autoxidation of benzoylformoin (BF), the end product of the former reaction. The effect of activated species of oxygen on rhodanese activity is also studied.

This paper is dedicated to the memory of Professor Eraldo Antonini, prematurely deceased 19 March, 1983

2. MATERIALS AND METHODS

Rhodanese was purified from bovine liver as in [5] and the activity assayed as in [6]. Crystalline enzyme, dissolved in 50 mM phosphate buffer (pH 7.8) and dialyzed overnight against the same buffer, was incubated at room temperature with α-ketoaldehydes or benzoylformoin in 1 ml final vol. The reaction time courses were followed by withdrawing at time intervals aliquots of the incubation mixtures which were assayed for enzymatic activity. Control experiments were run in parallel. To eliminate excess of reagents, the in-

Published by Elsevier Science Publishers B.V.

cubation mixture was sieved on Sephadex G-25 column. Sulfhydryl groups determination and electrophoresis on polyacrylamide gels with and without denaturing agents was as in [4,7]. Bovine Cu-Zn superoxide dismutase (SOD) was a generous gift of Dr G. Rotilio. Crystalline bovine liver catalase was obtained from Boehringer (Mannheim). Benzoylformoin (1,2,4-butanetrione3-hydroxy-1,4-diphenyl, BF) was prepared as in [8]. The yellow crystalline product (m.p. 180–182°C) was dissolved in absolute ethanol before use. Spectrophotometric measurements were carried out with Beckman ACTA IIIc or DU-8. An AMEL polarographic unit mod.461 was employed for measurements of the polarographic wave in the potential region where O_2^- is produced, by a dropping mercury electrode acting in an oxygen-saturated aqueous solution [9]. Oxygen consumption was measured polarographically at 25°C with a Clarke electrode using a YSI mod.53 biological oxygen monitor. Where anaerobic conditions were required, oxygen was removed from the reaction medium in a thunberg cuvette by repeated evacuation and flushing with nitrogen.

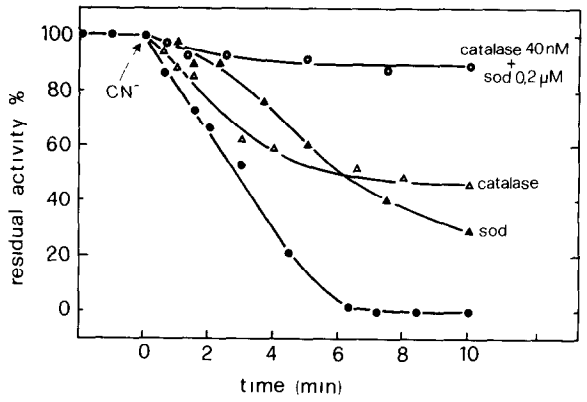

Fig.1. Effect of specific scavengers of oxygen reduction products on rhodanese inactivation by phenylglyoxal and cyanide. Standard reaction mixture: 25 μM rhodanese (sulfur-containing form), 50 μM phenylglyoxal in 50 mM phosphate buffer (pH 7.8) (final vol., 1 ml). Additions to the standard reaction mixture were: (●) none; (△) 40 nM catalase; (▲) 0.2 μM superoxide dismutase; (○) 40 nM catalase and 0.2 μM superoxide dismutase. Reactions were started by addition of 50 μM cyanide, then 5 μl aliquots were withdrawn and assayed for activity at indicated times.

3. RESULTS AND DISCUSSION

3.1. *Rhodanese inactivation by products of the reaction between phenylglyoxal and cyanide*

Sulfur–rhodanese is unaffected by stoichiometric amounts of α-ketoaldehydes compounds as PGO but, upon addition of a small excess of cyanide, the resulting sulfur-free enzyme is rapidly inactivated (fig.1). The absolute requirement of an excess of cyanide for enzyme inactivation indicates that the true inactivating agent could be a product of the reaction between PGO and cyanide, such as BF [1,3], originated by the condensation of two molecules of PGO catalyzed by cyanide. However, rhodanese inactivation caused by BF requires stoichiometric amounts of the reagent and occurs at a slower rate than in the presence of PGO and cyanide (fig.2); these findings raise doubts on the identity of BF as the true inactivating agent.

From similar experiments carried out in anaerobiosis we have observed that sulfur-free rhodanese is effectively protected against inactivation by either PGO or BF even in the presence of cyanide. Furthermore, polarographic measurements with Clarke electrode have shown that either the addition of cyanide to PGO or dilution of an alcoholic solution of BF in a buffered aqueous medium are followed by oxygen consumption. Quantitative determinations indicate that the amount of molecular oxygen consumed is stoichiometrically equivalent to the reagent added and that, in the presence of sulfur-free rhodanese equimolar with the reagent, the oxygen consumption parallels the inactivation. These results strongly support that the observed inactivation may be attributed to the reaction of sulfur-free enzyme with some of the intermediates in the reduction of molecular oxygen rather than to a direct interaction of the enzyme with the starting reagents.

The specific removal of O_2^- or hydrogen peroxide by addition of SOD or catalase to the incubation mixture protects to some extent rhodanese from inactivation. A similar partial protection is also achieved if O_2^- is trapped with 1 mM nitroblue tetrazolium (NBT). It is thus clear that O_2^- is implicated in such inactivation but it is not the only species involved and we cannot exclude an interaction of this radical or of its dismutation products with reactants formed in the incubation medium.

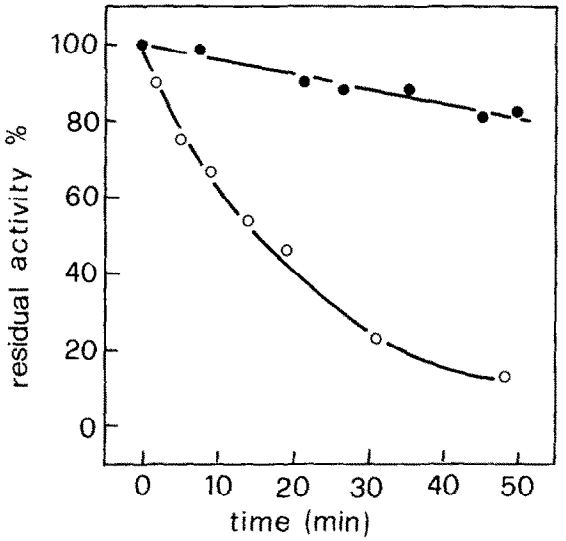

Fig.2. Time course of rhodanese inactivation by benzoylformoin. Sulfur-free rhodanese (30 μM) was incubated with an equimolar amount of benzoylformoin in the absence (○) or in the presence (●) of 80 nM catalase and 0.2 μM superoxide dismutase.

The simultaneous presence of SOD and catalase protects almost completely rhodanese against inactivation by PGO and cyanide or by BF (fig.1,2). This evidence confirms the hypothesis that oxygen radicals are involved in the enzyme inactivation.

Oxygen consumption and reduction of NBT, caused either by cyanidryn carbanion formed upon addition of cyanide to PGO or by aqueous dilution of BF, indicate that during autoxidation of these organic compounds, the release of O_2^- occurs. The reaction between this radical and its dismutation product hydrogen peroxide generates hydroxyl radical ($OH^\cdot$) which may represent the main agent responsible for rhodanese inactivation. In fact, only by specific removal of both superoxide radical and hydrogen peroxide with SOD and catalase enzyme protection is achieved.

3.2. *Effect of reduction products of oxygen on rhodanese activity*

To prove the involvement of oxygen reduction products in the inactivation of rhodanese by PGO or by BF, the enzyme was treated with known sources of oxygen radicals.

Superoxide radical was produced in a polarographic unit [9]. The effect of about 25 molar excess of O_2^- on 10 μM rhodanese in 100 mM borate buffer (pH 9.9) is shown in fig.3. Sulfur-rhodanese retains its sulfur transferring ability almost completely, while sulfur-free enzyme is progressively inactivated. In both cases loss of activity is prevented by addition of catalase (0.05 μM). Thus, the inactivating agent is hydrogen peroxide originated from partial dismutation of superoxide radicals.

The direct effect of hydrogen peroxide on enzyme activity was also tested. Sulfur-rhodanese was highly affected by an excess of this oxidizing agent [4]. Fig.4 exhibits the particular sensitivity of the sulfur-free form of rhodanese to a stoichiometric amount of hydrogen peroxide, in comparison with the relative stability of the sulfur-enzyme. Sulfur-free rhodanese is rapidly inactivated and the extent of inactivation is proportional to the amount of reagent added, up to 1 mol/mol of enzyme (fig.4, inset). This specific inactivation by hydrogen peroxide confirms the mechanism where the essential sulfhydryl group is involved in the ox-

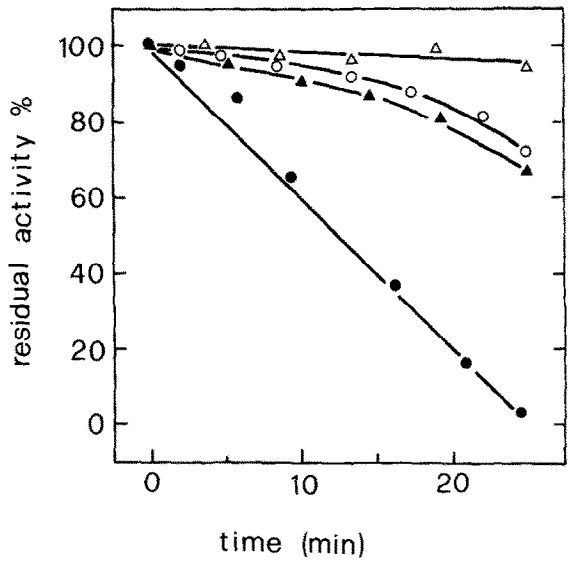

Fig.3. Time course of rhodanese inactivation by superoxide radical. The enzyme (10 μM) was incubated in a polarographic cell in which the source of O_2^- was a dropping mercury electrode acting in an O_2-saturated solution buffered at pH 9.9 with 100 mM borate and containing 0.6 mM triphenylphoshine oxide and 20 μM EDTA. Sulfur–enzyme (open symbols) and sulfur-free enzyme (solid symbols): in the presence (triangle) or in the absence (circle) of 50 nM catalase, respectively.

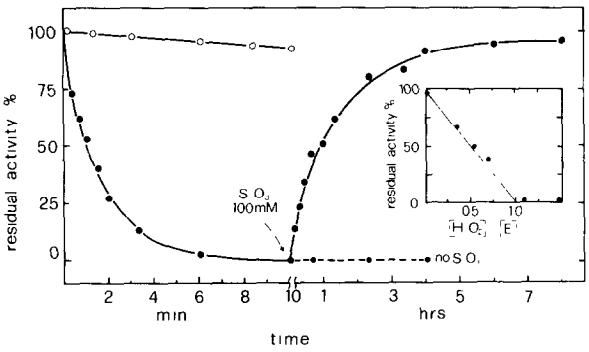

Fig.4. Effect of hydrogen peroxide on rhodanese activity. The enzyme (28 μM) was incubated with an equimolar amount of hydrogen peroxide in 50 mM phosphate buffer, 0.5 mM EDTA (pH 7.8): (○) sulfur-rhodanese; (●) sulfur-free rhodanese; (——→) addition of 100 mM thiosulfate; (···) represents the same experiment without thiosulfate. Inset: samples of sulfur-free enzyme were incubated with different H_2O_2 concentrations and tested for activity until no further inactivation occurred: the residual activity was then plotted against mol H_2O_2/mol enzyme. Experimental conditions as reported above.

idation reaction of rhodanese via the formation of a sulfenic group [4]. This derivative subsequently can react with a nearby sulfhydryl group to give a disulfide bridge. In fact, the sulfhydryl groups determination on the enzyme fully inactivated with 1 equiv. hydrogen peroxide indicates the loss of about 2 sulfhydryl groups among the 4 initially present in the native enzyme [10]. Gel electrophoresis analysis of the inactive enzyme, in the presence or absence of denaturing agents, showed the pattern typical of the native rhodanese. This result indicates that no intermolecular disulfide bridges occurred during rhodanese inactivation.

Complete removal of enzyme inactivation is achieved upon addition of 100 mM thiosulfate (fig.4). The reactivating effect of thiosulfate has been reported also for the enzyme inactivated by PGO and cyanide [1]. The recovery of enzyme activity upon treatment with thiosulfate indicates that residues essential for catalysis have not been irreversibly modified by hydrogen peroxide or by the products formed in the reaction between PGO and cyanide. The reduction of the disulfide bond by thiosulfate may proceed through nucleophilic attack of $S_2O_3^{2-}$ on one of the sulfur atoms [11]. Similarly to disulfide sulfitolysis, this attack could be facilitated by the presence of a positively charged group in the proximity of the disulfide bridge [1,12]. Then, a second molecule of thiosulfate can reduce the intermediate enzyme-sulfenyl-thiosulfate complex (E–S–S–SO_3^-), yielding a tetrathionate molecule and the active enzyme.

We conclude that the cyanide-promoted inactivation of rhodanese in the presence of ketoaldehydes is essentially due to the reaction of oxygen with organic products. The autoxidation of these products generates O_2^-, which is not a very reactive species by itself [13]. The conditions required to prevent enzyme inactivation indicate that OH· radical, produced from the O_2^- by the Haber–Weiss reaction, could be the most efficient inactivating agent. The failure of OH· scavengers, such as mannitol (150 mM), in significantly protecting the enzyme against inactivation may be attributed to the concomitant inactivating effect of hydrogen peroxide, which at pH 7.8 is rapidly produced by dismutation of O_2^- radicals, also in the absence of SOD. This possibility is confirmed by the finding that rhodanese activity is protected at a higher extent by the simultaneous presence of mannitol and catalase than by catalase alone.

The sensitivity of sulfur-free rhodanese towards hydrogen peroxide with respect to the native enzyme, is in agreement with previous findings of higher reactivity of the sulfur-free form of the enzyme with various inactivating agents [1,14,15]. The reported structural flexibility of the enzyme in this last form [16,17] may facilitate the formation of an intramolecular disulfide bond.

The restoration of activity via reduction with thiosulfate, a molecule diffusible through the mitochondrial membrane [18], suggests that this peroxidase-like activity of rhodanese may be of physiological importance in detoxication of hydrogen peroxide produced inside the mitochondria. The widespread occurrence of rhodanese in living cells leads us to suppose that such reaction could be particularly important for those organisms which lack catalases or peroxidases [19].

ACKNOWLEDGEMENTS

We thank Dr A. Finazzi Agrò for many useful discussions. This research was supported by grants from the 'Ministero della Pubblica Istruzione' and

the 'Consiglio Nazionale delle Ricerche', Rome (Italy).

REFERENCES

[1] Weng, L., Heinrikson, R.L. and Westley, J. (1978) J. Biol. Chem. 253, 8109–8119.
[2] Takahashi, K. (1977) J. Biochem. 81, 403–414.
[3] Weng, L. (1977) PhD. Dissertation, University of Chicago, Ch.3, pp.42–65, Chicago IL.
[4] Costa, M., Pecci, L., Pensa, B. and Cannella, C. (1977) Biochem. Biophys. Res. Commun. 78, 596–603.
[5] Horowitz, P. and De Toma, F. (1970) J. Biol. Chem. 245, 984–985.
[6] Sörbo, B.H. (1953) Acta Chem. Scand. 7, 1129–1136.
[7] Cannella, C., Costa, M., Pensa, B., Ricci, G., Pecci, L. and Cavallini, D. (1981) Eur. J. Biochem. 119, 491–495.
[8] Karrer, V.P. and Segesser, A.V. (1935) Helv. Chim. Acta 18, 273–275.
[9] Rigo, A., Viglino, P. and Rotilio, G. (1975) Anal. Biochem. 68, 1–8.
[10] Sörbo, B.H. (1963) Acta Chem. Scand. 17, S107–109.
[11] Szczepkowki, T.W. (1958) Nature 182, 934–935.
[12] Chan, W. (1968) Biochemistry 7, 4247–4254.
[13] Fridovich, I. (1976) in: Free Radicals in Biology (Pryor, W.A. ed) pp.239–277, Academic Press, New York.
[14] Pensa, B., Costa, M., Pecci, L., Cannella, C. and Cavallini, D. (1977) Biochim. Biophys. Acta 484, 368–374.
[15] Horowitz, P. and Criscimagna, N.L. (1982) Biochim. Biophys. Acta 702, 173–177.
[16] Wasylewski, Z. and Horowitz, P. (1982) Biochim. Biophys. Acta 701, 12–18.
[17] Blicharska, B., Kołoczek, H. and Wasylewski, Z. (1982) Biochim. Biophys. Acta 708, 326–329.
[18] Crompton, M., Palmieri, F., Capano, M. and Quagliariello, E. (1974) FEBS Lett. 46, 247–250.
[19] Ramasarma, T. (1982) Biochim. Biophys. Acta 694, 69–93.