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Primary and post-irradiation inactivation of the sulfhydryl enzyme malate synthase: correlation of protective effects of additives

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The presence of additives during X-irradiation of malate synthase led to radioprotective effects against primary and post-irradiation inactivation. Pronounced effects were provided by typical scavengers, sulfhydryl reagents and specific ligands (substrates, products, analogues). The results show that scavenging and specific protection are responsible for the protective efficiency of additives. Scavengers delete noxious species formed during irradiation or post-radiationem. Sulfhydryl reagents may act as repair substances. Specific ligands protect the active site of the enzyme and the essential sulfhydryls; specific protection is more pronounced post-radiationem. Ligands and sulfhydryl reagents may additionally act as scavengers. A cumulative index for the protective power of additives against both sorts of inactivation was established.

Malate synthase; X-irradiation; Inactivation; Additive; Protection; Correlation

1. INTRODUCTION

Malate synthase of the glyoxylate cycle catalyzes the Mg^{2+}-dependent condensation of glyoxylate and CoASAc to form L-malate and CoASH. Previous experiments with the yeast enzyme (M_r = 185 000) showed that the reaction follows a sequential random mechanism [1]. Results indicated the existence of a nucleotide-binding site for CoASAc on the enzyme and the formation of a chelate between enzyme, Mg^{2+} and glyoxylate [1,2]. Substrate analogues like pyruvate, oxaloacetate, α-ketobutyrate, glycollate were shown to bind to the enzyme and to inhibit it strictly competitively for glyoxylate [1–3], while lactate, e.g. obviously does not bind to the enzyme [4]. L-Malate was shown to be a weak non-competitive inhibitor [3]; both L-, D- and DL-malylcoenzyme A can be hydrolyzed by the enzyme [2]. Conformational changes of the enzyme upon ligand binding (glyoxylate, CoASAc, pyruvate, CoASAc + pyruvate) have been demonstrated [5–9]; these as well as isotope exchange reactions are consistent with a (second) substrate-dependent change from a catalytically inactive to an active conformation [1].

The yeast enzyme turned out to be an attractive example for the investigation of structural and functional changes as a consequence of X-irradiation [6,10–18] for several reasons: e.g. the enzyme has sulfhydryls essential for activity (1 per subunit), the enzymic test is simple and can be performed quickly (90 s), and the solution structure (oblate shape) is well characterized. The enzyme sulfhydryls turned out to be crucial for radiation-induced inactivation and aggregation [11]. The sulfhydryl agent DTT was found to protect the enzyme during X-irradiation against aggregation and

Dedicated to Professor Dr Erwin Schauenstein on the occasion of his 70th birthday

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Abbreviations: a.r., ante radiationem; p.r., post radiationem; CoASH, coenzyme A; CoASAc, acetyl-coenzyme A; DTT, dithiothreitol; catalase (EC 1.11.1.6); malate synthase (EC 4.1.3.2); SOD, superoxide dismutase (EC 1.15.1.1)

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inactivation [6,10–13]. Post-irradiation addition of DTT to inactivated enzyme resulted in a considerable repair of enzymic activity [11–13]. In aqueous solution in the presence of oxygen inactivation of the enzyme during irradiation is mainly caused by ·OH radicals (directly of via secondary radicals), post-irradiation inactivation being mainly due to the action of the more stable H_2O_2 [12, 13].

The present paper reports on systematic investigations of inactivation and protection during irradiation and in the post-irradiation phase. Experiments were performed in the absence/presence of a variety of different sorts of a.r. additives: typical scavengers, sulfhydryl reagents, buffer substances, inorganic salts, substrates, products, substrate and product analogues. In the case of the enzyme irradiated in the absence of a.r. additives, the influence of some p.r. additives on p.r. inactivation has been studied additionally.

2. MATERIALS AND METHODS

2.1. *Materials*

Malate synthase (40–50 IU/mg) was isolated from baker's yeast in electrophoretically pure form as in [1]. CoASAc for the enzymic tests was prepared according to [19]. Catalase from bovine liver, SOD from bovine erythrocytes, CoASAc, CoASH, and oxaloacetic acid were purchased from Boehringer, Mannheim. Na formate, glyoxylic acid, NaCl, $MgCl_2$ and Tris were obtained from Merck, Darmstadt, Na pyruvate and Na lactate from Serva, Heidelberg, L-, D- and DL-malic acid from Roth, Karlsruhe, Na α-ketobutyrate and glycolic acid from Sigma, Munich, and reduced and oxidized forms of DTT from Calbiochem, Luzern. All other reagents were of A-grade purity. Quartz-bidistilled water was used throughout.

2.2. *Solutions*

5 mM Tris-HCl buffer (pH 8.1), containing 10 mM $MgCl_2$, 1 mM MgK_2EDTA and 0.2 mM DTT, was used as a standard buffer. Malate synthase was dialyzed at 4°C against this buffer. Concentrations of enzymes and additives were determined spectrophotometrically or gravimetrically. For irradiation experiments, carefully prepared stock solutions of malate synthase and additives (all adjusted to pH 8.1) were mixed to give an enzyme concentration of 0.5 mg/ml and the concentrations of additives listed in table 1.

2.3. *X-irradiation*

Solutions were X-irradiated with the unfiltered radiation from a Philips PW 2253/11 X-ray tube (Cu, 50 kV, 30 mA) in the microcell described in [10,11]. Air-saturated solutions were irradiated in the sealed cell ($V = 240\ \mu l$) at 4°C with 2 kGy (dose rate 180 Gy/min as determined by Fricke dosimetry) and stored afterwards at the same temperature in 1 ml plastic tubes.

2.4. *Enzymic assay*

The assay was performed at 20°C as described [1,2] using a Zeiss PMQ II spectrophotometer. Aliquots of the samples (1–20 μl) were used in the test as outlined in [11]. Each test was performed at least 3 times; accuracy of activities was better than 5%. The irradiated solutions were tested shortly after stopping irradiation ($t = 0$), and 30 h afterwards; unirradiated references were treated in a similar manner. In most cases the activity of the references turned out to be almost unaffected by the presence of a.r. additives. In some cases (samples 12, 17–22, 24, 26) spontaneous inactivation occurred, which was taken into account in the determination of residual activities, A_r; similar A_r values, however, were obtained in the simultaneous presence of 100 mM a.r. $MgCl_2$, which effectively suppressed spontaneous inactivation.

2.5. *Post-irradiation repair*

A concentrated DTT solution was added to the irradiated solutions at $t = 0$ (final DTT concentration: 10 mM). The extent of repair was checked 30 h later. The unirradiated references were treated analogously; deviations were taken into account.

2.6. *Post-irradiation treatment*

To aliquots of the irradiated sample no. 1 (without a.r. additives) some p.r. additives were added at $t = 0$ (samples 2–5) and activities were monitored 30 h later.

2.7. *Calculation of parameters*

Residual activities, A_r, were derived from the ratio of the activities of irradiated and unirradiated samples. The A_r values at $t = 0$ render information on the extent of primary inactivation. Assuming an exponential decay of activity in the p.r. phase (cf. [12,13]), apparent first-order rate constants, k', may be deduced from the A_r values at $t = 0$ and $t = 30$ h p.r. The k' values may be regarded as a suitable measure of the progress of post-irradiation inactivation [12]. In order to allow a quantitative comparison of the protective efficiency of the various a.r. additives against primary and post-irradiation inactivation, both quantities (A_r values at $t = 0$ and k' values) may be normalized with respect to the corresponding values of the enzyme irradiated in the absence of a.r. and p.r. additives:

$$p_{A_r} = \frac{A_r^{t=0}\ \text{(with additives)} - A_r^{t=0}\ \text{(without additives)}}{100 - A_r^{t=0}\ \text{(without additives)}}$$

$$p_{k'} = \frac{k'\ \text{(without additives)} - k'\ \text{(with additives)}}{k'\ \text{(without additives)}}$$

The values for p_{A_r} and $p_{k'}$ generally vary between 0 (0% protection) and 1 (100% protection); negative values indicate an enhancement of inactivation; values >1 reflect the occurrence of additional repair phenomena. The protective power, P, defined as the geometric mean

$$P = (p_{A_r} \cdot p_{k'})^{0.5},$$

is an appropriate quantitative measure of the protective efficiency of a.r. additives against both primary and p.r. inactivation. The protective efficiency of the various a.r. additives may also be followed qualitatively from their rank (S_{A_r},$S_{k'}$,S_P) in the corresponding series (cf. table 1).

Table 1

Composition of samples, primary and post-irradiation inactivation of malate synthase upon 2 kGy X-irradiation and protective power of a.r. or p.r. additives

Samples	Additives a.r. (p.r.)	Primary inactivation			Post-irradiation inactivation			Protective power	
		$A_r^{t=0}$ (%)	p_{A_r}	S_{A_r}	k' (h^{-1}) ($\times 10^2$)	$p_{k'}$	$S_{k'}$	P	S_P
1	none	3.4	0	24	3.53	0	23	0	23
2	none (100 mM NaCl)	3.4	0		1.17	0.67		0	
3	none (100 mM $MgCl_2$)	3.4	0		0.33	0.91		0	
4	none (100 mM Na glyoxylate)	3.4	0		0.32	0.91		0	
5	none (100 mM Na formate)	3.4	0		2.31	0.35		0	
6	100 mM Na formate	71.6	0.71	6	0.69	0.80	11	0.75	8
7	4 μM SOD	8.5	0.05	20	5.17	−0.46	24		
8	550 nM catalase	3.8	0.004	22	3.10	0.12	22	0.02	21
9	100 mM Na formate + 4 μM SOD	85.6	0.85	2	1.51	0.57	15	0.70	11
10	100 mM Na formate + 550 nm catalase	79.0	0.78	4	0.38	0.89	8	0.84	2
11	5 mM DTT	90.1	0.90	1	0	1.00	1	0.95	1
12	5 mM DTT oxidized	3.5	0.001	23	2.20	0.38	20	0.02	21
13	100 mM Tris-HCl	7.1	0.04	21	1.80	0.49	19	0.14	20
14	100 mM NaCl	11.1	0.08	19	1.75	0.50	18	0.20	18
15	100 mM $MgCl_2$	12.8	0.10	18	1.73	0.51	16	0.22	17
16	100 mM Na glyoxylate	69.7	0.69	7	0.06	0.98	3	0.82	3
17	5 mM Na CoASAc	22.7	0.20	16	0.67	0.81	10	0.40	16
18	100 mM Na L-malate	62.6	0.61	11	0.03	0.99	2	0.78	5
19	100 mM Na D-malate	64.1	0.63	10	0.34	0.90	7	0.75	8
20	100 mM Na DL-malate	69.5	0.68	8	0.20	0.94	4	0.80	4
21	50 mM Na L-malate + 50 mM Na D-malate	66.4	0.65	9	0.30	0.92	5	0.77	6
22	5 mM Na CoASH	84.0	0.83	3	1.73	0.51	16	0.65	13
23	100 mM Na pyruvate	19.1	0.16	17	2.99	0.15	21	0.16	19
24	5 mM Na CoASAc + 100 mM Na pyruvate	60.0	0.59	12	0.33	0.91	6	0.73	10
25	100 mM Na α-ketobutyrate	39.4	0.37	15	0.86	0.76	13	0.53	14
26	100 mM Na oxaloacetate	75.1	0.74	5	0.74	0.79	12	0.77	6
27	100 mM Na glycollate	39.8	0.38	14	1.05	0.70	14	0.51	15
28	100 mM Na lactate	57.3	0.56	13	0.50	0.86	9	0.69	12

Parameters are defined in section 2. For comparison the A_r values for formate, SOD and catalase have been taken from a previous paper [13]

3. RESULTS AND DISCUSSION

Different mixtures of malate synthase with a.r. additives were X-irradiated and analyzed for enzymic activity. The composition of 28 characteristic samples and the results are given in table 1. Some representative experiments are outlined in fig.1.

3.1. *Primary inactivation*

Strong protective effects against primary inactivation were provided by the sulfhydryl compounds DTT and CoASH, less markedly by typical $^{\cdot}OH$ radical scavengers (e.g. formate) and some substrates, products and analogues (oxaloacetate, glyoxylate, malate, CoASAc + pyruvate), and were less expressed by lactate. No significant difference between L-, D- and DL-malate was noted. Only weak protective effects were achieved by inorganic salts or the buffer components.

3.2. *Repair of primary inactivation*

The addition of DTT to irradiated samples (cf. fig.1) in most cases led to a more or less pronounced increase in residual activities: cf., e.g. the enzyme without additives of in the presence of some a.r. additives (oxidized DTT, catalase, SOD, pyruvate, inorganic salts, CoASAc, buffer substances). The extent of repair was rather low with DTT, formate, glyoxylate, L-, D- and DL-malate,

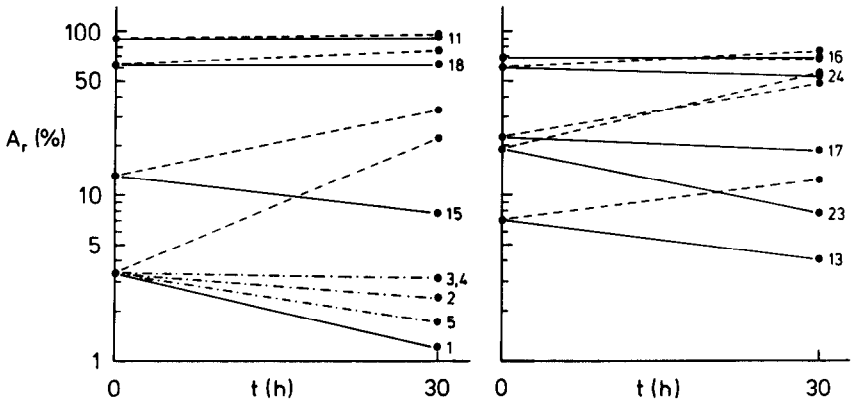

Fig.1. Residual activity, A_r, of samples 1–5, 11, 13, 15–18, 23 and 24 as a function of time, t, after stopping of 2 kGy X-irradiation. The composition of the samples is given in table 1. (——) Post-irradiation inactivation; (– – –) repair by p.r. DTT; (–·–·–) inhibition of p.r. inactivation by p.r. additives.

CoASH, CoASAc + pyruvate and some analogues (glycollate, oxaloacetate) and lactate. Fig.1 clearly shows that the percentual repairs are more pronounced in the absence of effective a.r. protectives.

3.3. *Post-irradiation inactivation*

The k' values depend strongly on the presence of a.r. additives. A pronounced suppression of p.r. inactivation is provided by DTT, glyoxylate, L-, D- and DL-malate, CoASAc + pyruvate, lactate, CoASAc, formate. Glyoxylate and $MgCl_2$ proved to be also effective p.r. additives, when added to the enzyme irradiated without a.r. additives, while p.r. formate and p.r. NaCl were less effective.

3.4. *Correlation of protective effects*

As follows from an inspection of the P and S_P values (table 1), the most potent radioprotective substances against both primary and post-irradiation inactivation are DTT, glyoxylate, malate, oxaloacetate, formate, CoASAc + pyruvate and lactate. This may also be concluded from fig.2, correlating p_{A_r} and $p_{k'}$. The a.r. additives representing good protectives against primary inactivation are located in the uppermost part of the plot, additives which effectively impede p.r. inactivation are located in the right-hand part. The protective efficiency of most a.r. additives against p.r. inactivation exceeds the efficiency against primary inactivation. Some extreme examples in this context are, e.g. the inorganic salts, Tris-HCl, and oxidized DTT.

As may be seen from fig.2, a comparison of the weak protective efficiencies for CoASAc and/or pyruvate on the one hand with the strong protection provided by CoASAc + pyruvate on the other strongly suggests a specific shielding of the active site of the enzyme. The position of the substrate glyoxylate and of the product L-malate close to the right-hand margin of the drawing point in the same direction.

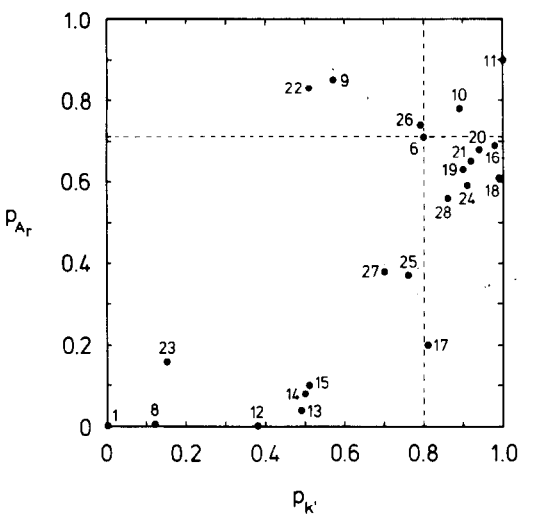

Fig.2. Correlation of protective efficiencies, p_{A_r} and $p_{k'}$, obtained from data ($A_r^{t=0}$,k') characterizing primary and post-irradiation inactivation of malate synthase. The lowest and highest protective efficiencies are located in the lower left and upper right corner of the plot, respectively. The p_{A_r} and $p_{k'}$ values corresponding to the typical ·OH scavenger formate are marked by dashed lines.

4. CONCLUSIONS

The above results clearly show that 3 types of protectives are most effective against primary and post-irradiation inactivation of malate synthase:
(i) Typical scavengers (e.g. $^{\cdot}OH$ scavenger formate). The use of enzymes (SOD or catalase in catalytic amounts) as sole protectives during irradiation seems to be problematic, since they may also be damaged. The simultaneous presence of formate and the $O_2^{\cdot-}$ scavenger SOD, however, gave rise to an enhanced effect against primary inactivation as compared to formate alone, while formate together with H_2O_2 scavenger catalase impeded p.r. inactivation to a higher degree than formate.
(ii) Reagents with free sulfhydryls (DTT, CoASH).
(iii) Substrates, products and some analogues: especially glyoxylate, malate, and analogues like oxaloacetate, and the combination CoASAc + pyruvate.

Inorganic salts (NaCl, $MgCl_2$) and buffer substances (Tris-HCl) only provided weak protective effects.

Summarizing these results, we may state that both scavenging of the deleterious radical and non-radical species, formed during irradiation or p.r., as well as specific protection (of the catalytic or other sensitive sites of the enzyme) are involved in the protection mechanism. The sulfhydryl reagents maintain the intactness of the enzyme sulfhydryls, the specific ligands shielding and protecting the active site and the essential sulfhydryls. Both types of protectives may additionally act as scavengers (cf. [15,20]). The specific protection is obviously more pronounced in the p.r. phase, probably due to the fact that there are only minor contributions from radical damage [13] and protection of essential sulfhydryls against attack by H_2O_2 molecules becomes predominant.

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