X-Ray Induced Inactivation of the Sulfhydryl Enzyme Malate Synthase in the Presence of Various Additives
Probing the Extent of Primary and Post-Irradiation Inactivation and Repair by Rapid Screening on the Microlevel*

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The sulfhydryl enzyme malate synthase was inactivated by X-irradiation in air-saturated aqueous solution, in the absence or presence of a variety of additives (thiols, antioxienzymes, typical radical scavengers, inorganic salts, buffer components, substrates, products, analogues). Radiation-induced changes of enzymic activity were registered immediately after stop of irradiation and in the post-irradiation period. Repair experiments were initiated by post-irradiation addition of dithiothreitol. Additionally, post-irradiation inactivation was modulated by some further additives. Probing the extent of primary and post-irradiation inactivation and repair was accomplished effectively by screening experiments on the microlevel, and by derivation of normalized efficiency parameters which allowed quick comparisons of the various additives with respect to their protective and repair-promotive efficiencies. Correlations between the efficiency parameters were studied by means of binary and ternary diagrams. Most of the substances added before irradiation were found to protect the enzyme against primary and post-irradiation inactivation and to increase the reparability of the enzyme by dithiothreitol, the extent of the effects depending on the nature (and concentration) of the additives used. Our results indicate that both specific protection (by substrates, products, analogues, and by sulfhydryl agents) and scavenging are responsible for the radioprotective efficiencies of the additives.

Introduction

The investigation of sulfur-containing biomolecules has a long tradition in radiation biology (*cf.* [1, 2]); inactivation studies of sulfhydryl enzymes in aqueous solution turned out to be of particular interest [2, 3]. Both structural and functional changes of the sulfhydryl enzyme malate synthase as a consequence of X-irradiation have been studied previously [4–15].

Malate synthase mediates the condensation of glyoxylate and CoASAc to form (L)-malate and CoASH; the reaction exhibits a requirement for Mg^{2+}; results indicate the formation of a complex between Mg^{2+} and the substrates on the enzyme [16, 17]. The enzyme from baker's yeast has been characterized thoroughly by biochemical [16–20] and physico-chemical [4, 19, 21–24] techniques: the trimeric enzyme has a molecular weight of about 185,000 [19, 22], is of oblate shape [22], and has sulfhydryls essential for activity (1 per subunit) [6]. The substrates bind in sequential random order [19]. Various substrate analogues (*e.g.,* pyruvate, oxaloacetate, α-ketobutyrate, glycollate) bind to the enzyme and inhibit it competitively for glyoxylate [16–19], while (L)-lactate, *e.g.,* did not show comparable effects [18]. Different conformational changes of the enzyme have been demonstrated upon binding glyoxylate (or pyruvate), CoASAc, or CoASAc + pyruvate [21–23], respectively. Consistent with these observations, sophisticated isotope techniques showed that the condensation catalyzed by the enzyme follows a stepwise path [20].

Abbreviations and enzymes: 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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Previous studies of malate synthase [4–15] have shown that X-irradiation in aqueous solution in the presence of oxygen may lead to a variety of damages: modification of amino acids (*e.g.*, oxidation of sulfhydryls), inactivation (continuing p.r.), subunit cross-linking, enzyme aggregation, fragmentation, unfolding etc. In accordance with the indirect action of radiation, a linear relation between D^{1}_{37} and enzyme concentration was obtained [9, 25]. Activity of the damaged enzyme could be restored partly by p.r. addition of DTT [6–8, 14]. In aqueous solution in the presence of oxygen, enzyme inactivation is mainly caused by ·OH radicals (directly or *via* secondary radicals), post-irradiation inactivation is primarily due to the action of H_2O_2.

To probe the modulation of the X-ray induced inactivation of the enzyme by various additives, we have performed a series of screening experiments on the microlevel. In continuation of our previous experiments [8], we used air-saturated aqueous solutions, thus mimicking quasi-physiological conditions (*cf.* [26]). Primary and post-irradiation effects as well as the restoration of enzymic activity (repair) were investigated. In order to compare quantitatively the additives with respect to their radioprotective efficiency against primary and p.r. inactivation, and their influence on the reparability of the enzyme by DTT, we established appropriate parameters. Attempts were made to correlate the parameters characterizing the protective and repair-promotive efficiencies.

Materials and Methods

Materials

Malate synthase (40–50 IU/mg) was isolated from baker's yeast as described [19]. CoASAc for the enzymic tests was prepared according to [27]. 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-(L)-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.

Solutions

For biochemical reasons, a 5 mM Tris/HCl buffer pH 8.1, containing 10 mM $MgCl_2$, 1 mM MgK_2EDTA and 0.2 mM DTT, was used. For irradiation experiments, carefully prepared stock solutions of malate synthase and of additives (all adjusted to pH 8.1) were mixed to give an enzyme concentration of 0.5 mg/ml (= 2.7 μM) and the final concentrations of a.r. additives listed in Table I.

Experimental design of screening experiments

Rapid screening of a large number of (a.r. and p.r.) additives necessitated a special experimental design: performance of rapid experiments on the microlevel; use of only one enzyme and generally one additive concentration; irradiation of air-saturated solutions at one dose (no time-consuming gassing procedures); activity measurements at only a few sampling times, using a sophisticated time-schedule allowing synchronous measurements; determination of simple parameters, enabling the characterization and the comparison of primary and p.r. inactivation and repair under the given experimental restrictions. Enzyme concentration and radiation dose had to be chosen in such a way that additives of quite different efficiencies could be screened without changing experimental conditions.

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 earlier [5, 6]. Air-saturated solutions were irradiated in the sealed cell (V = 240 μl) 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.

Enzymic assay

The enzyme was assayed at 20 °C as described [19], using a Zeiss PMQ II spectrophotometer. Further details are outlined in [6, 8]. The irradiated solutions were tested immediately after irradiation (t = 0), and 30 h later (t = 30), using small aliquots (1–20 μl) in the enzymic test; unirradiated refer-

Table I. Final concentrations of a.r. additives.

Sample No.	a.r. Additives	Sample No.	a.r. Additives
1	None	22	100 mM NaCl
2	0.4 μM SOD	23	200 mM NaCl
3	55 nM Catalase	24	400 mM NaCl
4	0.4 μM SOD + 55 nM Catalase	25	50 mM $MgCl_2$
5	4.0 μM SOD	26	100 mM $MgCl_2$
6	550 nM Catalase	27	200 mM $MgCl_2$
7	4.0 μM SOD + 550 nM Catalase	28	100 mM NaCl + 50 mM $MgCl_2$
8	10 mM Na-Formate	29	100 mM NaCl + 100 mM $MgCl_2$
9	10 mM Na-Formate + 0.4 μM SOD	30	100 mM Na-Glyoxylate
10	10 mM Na-Formate + 55 nM Catalase	31	5 mM Na-CoASAc
11	10 mM Na-Formate + 0.4 μM SOD + 55 nM Catalase	32	100 mM Na-(L)-Malate
12	100 mM Na-Formate	33	100 mM Na-(D)-Malate
13	100 mM Na-Formate + 0.4 μM SOD	34	100 mM Na-(DL)-Malate
14	100 mM Na-Formate + 55 nM Catalase	35	50 mM Na-(L)-Malate + 50 mM Na-(D)-Malate
15	100 mM Na-Formate + 0.4 μM SOD + 55 nM Catalase	36	5 mM Na-CoASH
16	100 mM Na-Formate + 4.0 μM SOD	37	100 mM Na-Pyruvate
17	100 mM Na-Formate + 550 nM Catalase	38	5 mM Na-CoASAc + 100 mM Na-Pyruvate
18	100 mM Na-Formate + 4.0 μM SOD + 550 nM Catalase	39	100 mM Na-α-Ketobutyrate
19	5 mM DTT	40	100 mM Na-Oxaloacetate
20	5 mM DTT oxidized	41	100 mM Na-Glycollate
21	100 mM Tris/HCl	42	100 mM Na-(L)-Lactate

Experimental results for samples Nos. 1 and 19–42 are given in Tables III and IV of this paper. For correlation plots (Figs. 1 and 2) some data have been adopted from a previous paper [8] (samples Nos. 2–18: the numbering of these samples is the same as in [8]).

ences were treated similarly. In a few cases a correction allowing for spontaneous inactivation was adopted (*cf.* [14]).

Post-irradiation repair

To study the repair behaviour, a concentrated DTT solution was added to irradiated solutions at $t = 0$ to give a final concentration of 10 mM DTT. Activities were determined 3 and 30 h later. Unirradiated references were treated analogously. Generally the repair was nearly complete already after 3 h (*cf.* [7]). For calculation of parameters, we used the value determined at 30 h to guarantee completeness of repair. With a few samples, a pronounced repair at 3 h, however, was found to be followed by a considerable inactivation at $t = 30$ h.

Post-irradiation treatment

With some samples a modulation of p.r. inactivation was performed by adding concentrated solutions of p.r. additives at $t = 0$ to give the final concentrations outlined in Table II. Activities were controlled 30 h later.

Table II. Final concentrations of p.r. additives.

Sample No.	p.r. Additives
1b	100 mM NaCl
1c	100 mM $MgCl_2$
1d	100 mM Na-Glyoxylate
1e	90 mM Na-Formate
1f	0.33 μM SOD + 45 nM Catalase
8b	0.36 μM SOD
8c	50 nM Catalase
8d	0.33 μM SOD + 45 nM Catalase
12b	0.36 μM SOD
12c	50 nM Catalase
12d	0.33 μM SOD + 45 nM Catalase
12e	3.6 μM SOD
12f	500 nM Catalase
26b	100 mM Na-Glyoxylate

Experimental results for samples Nos. 1b–d and 26b are given in Table IV of this paper. For the correlation plot shown in Fig. 1 some data have been adopted from a previous paper [8] (samples Nos. 1e–f, 8b–d, 12b–f).

Calculation of parameters

The extent of primary inactivation followed directly from the residual activities after stop of irradiation ($A_r^{t=0}$ in %). Post-irradiation inactivation

was expressed by normalized residual activities (in %) at $t = 30$ h

$$A_n^{t=30} = 100 \cdot A_r^{t=30}/A_r^{t=0}. \quad (1)$$

The gain of DTT-repair, Q, was obtained from residual activities of repaired samples, $A_R^{t=30}$, according to

$$Q = A_R^{t=30}/A_r^{t=0}. \quad (2)$$

The above mentioned parameters are independent of any kinetic assumptions. As in previous papers [8, 13], the primary and p.r. inactivation behaviour was characterized by some further parameters, which, however, necessitated the assumption of exponential decays of activity. The approximate validity of this assumption was tested for some samples [5; 7, 8].

Inactivation doses D_{37}^t for total (= repairable + non-repairable) inactivation were calculated from $A_r^{t=0}$ by assuming an exponential dose-effect curve. Similarly, inactivation doses $D_{37}^{n.r.}$ for non-repairable inactivation were derived from $A_R^{t=30}$-values. Apparent half-lives, τ', were calculated from $A_n^{t=30}$, assuming an exponential decay of activity in the p.r. phase (*cf.* [13]).

Normalized efficiency parameters

In order to allow a quantitative comparison of the various a.r. additives with respect to their protective efficiencies against primary and p.r. inactivation, appropriate normalized quantities were derived from residual activities, $A_r^{t=0}$ and $A_n^{t=30}$:

$$p_{A_r} = \frac{A_r^{t=0}\,(\text{with additives}) - A_r^{t=0}\,(\text{without additives})}{100 - A_r^{t=0}\,(\text{without additives})} \quad (3)$$

$$p_{A_n} = \frac{A_n^{t=30}\,(\text{with additives}) - A_n^{t=30}\,(\text{without additives})}{100 - A_n^{t=30}\,(\text{without additives})}. \quad (4)$$

As a consequence of the normalization to the sample without additives, the values for p_{A_r} and p_{A_n} generally vary between 0 and 1, reflecting 0 or 100% protection against primary and p.r. inactivation, respectively. Negative values indicate an enhancement of inactivation caused by a.r. additives, as compared to the sample without additives. Values > 1 may be a hint for additional repair phenomena caused by a.r. additives. It should be noted, that the protective efficiencies, p_{A_r} and p_{A_n}, do not depend on the assumption of exponential decays.

For the comparison of a.r. additives with regard to their promotive efficiency for DTT-repair, a normalized quantity was derived from $A_R^{t=30}$ and $A_r^{t=0}$:

$$p_{A_R} = \frac{A_R^{t=30} - A_r^{t=0}}{100 - A_r^{t=0}}. \quad (5)$$

This formula applies to samples both in the absence or presence of a.r. additives. In contrast to the protective efficiencies, p_{A_r} and p_{A_n}, the promotive efficiency for DTT-repair, p_{A_R}, is not normalized to the sample without a.r. additives (which itself is highly repairable). Hence, all values for p_{A_R} are positive. The values for p_{A_R} generally lie between 0 and 1, corresponding to 0 and 100% restoration of enzymic activity, respectively. Negative values would indicate an inactivation caused by p.r. DTT, values > 1 may occur as a consequence of additional repair phenomena caused by a.r. additives.

A normalization of the promotive efficiency for DTT-repair in analogy to the parameters p_{A_r} and p_{A_n}, *i.e.* a normalization with respect to the sample without a.r. additives, might be performed according to:

$$p_R = \frac{p_{A_R}\,(\text{with additives}) - p_{A_R}\,(\text{without additives})}{1 - p_{A_R}\,(\text{without additives})}. \quad (6)$$

This parameter, however, would become negative for all a.r. additives promoting DTT-repair less pronounced than the sample without a.r. additives.

To correlate the parameters p_{A_r}, p_{A_n}, and p_{A_R}, two-dimensional plots of p_{A_n} *vs.* p_{A_r}, p_{A_R} *vs.* p_{A_r}, and p_{A_R} *vs.* p_{A_n} can be drawn.

A ternary diagram has to be used to correlate simultaneously the efficiency parameters characterizing primary inactivation, p.r. inactivation, and extent of repair. For this purpose the parameters p_{A_r}, p_{A_n}, and p_{A_R}, must be renormalized:

$$p_{A_r}^* = p_{A_r} / (p_{A_r} + p_{A_n} + p_{A_R}) \quad (7)$$

$$p_{A_n}^* = p_{A_n} / (p_{A_r} + p_{A_n} + p_{A_R}) \quad (8)$$

$$p_{A_R}^* = p_{A_R} / (p_{A_r} + p_{A_n} + p_{A_R}) \quad (9)$$

$$p_{A_r}^* + p_{A_n}^* + p_{A_R}^* = 1. \quad (10)$$

For the renormalization only efficiency parameters > 0 can be used; this condition rules out the use of p_R.

Propagation of errors

Each enzymic test was performed at least 3 times. Additionally, some experiments were re-

peated with different sets of samples. The standard deviation of calculated residual activities generally was $< 5\%$ of A_r. The propagation of these errors was investigated for all parameters calculated (*cf.* Tables III and IV). This was facilitated by a computer program which was used for the calculation of all parameters.

Results

Malate synthase was X-irradiated in the absence or presence of a.r. additives and treated with p.r. DTT or other p.r. additives after stop of irradiation. The composition of some characteristic samples is given in Tables I and II. The results characterizing primary and post-irradiation inactivation and repair behaviour are outlined in Tables III and IV.

Primary inactivation

Table III shows $A_r^{t=0}$-values, and quantities derived therefrom (p_{A_r}, D_{37}^t). As follows from the tabulated values, strong protective effects against primary inactivation were provided by sulfhydryl compounds (DTT, CoASH), by some substrates, products, and analogues (glyoxylate, malate, CoASAc + pyruvate, oxaloacetate), and by lactate. Weak effects were provided by CoASAc, pyruvate, and by the inorganic salts, NaCl and $MgCl_2$, and by Tris/HCl. No effect was observed for DTT oxidized.

Repair of primary inactivation

In most cases the addition of p.r. DTT led to a considerable increase of enzyme activity (Table III: $A_R^{t=30}$). It should be emphasized, that the parameters Q and p_{A_R}, characterizing the extent

Table III. Primary inactivation of malate synthase upon 2 kGy X-irradiation in the presence of various a.r. additives and repair by DTT.

Sample No.[a]	Primary inactivation		Repair			Inactivation dose		
	$A_r^{t=0}$ [%]	p_{A_r}	$A_R^{t=30}$[b] [%]	Q	p_{A_R}[c]	D_{37}^t [kGy]	$D_{37}^{n.r.}$ [kGy]	$D_{37}^t/D_{37}^{n.r.}$
1	3.4 ± 0.1	0.00 ± 0.00	22.0 ± 1.5	6.5 ± 0.5	0.19 ± 0.02	0.59 ± 0.01	1.32 ± 0.06	0.45 ± 0.02
19	90.1 ± 2.6	0.90 ± 0.03	94.5 ± 3.2	1.05 ± 0.05	0.44 ± 0.44	19.2 ± 5.4	35 ± 21	0.54 ± 0.36
20	3.5 ± 0.4	0.00 ± 0.00	47.4 ± 1.6	13.5 ± 1.7	0.45 ± 0.02	0.60 ± 0.02	2.68 ± 0.12	0.22 ± 0.01
21	7.1 ± 0.5	0.04 ± 0.01	12.3 ± 1.0	1.7 ± 0.2	0.06 ± 0.01	0.76 ± 0.02	0.96 ± 0.04	0.79 ± 0.04
22	11.1 ± 0.4	0.08 ± 0.00	30.7 ± 1.8	2.8 ± 0.2	0.22 ± 0.02	0.91 ± 0.02	1.70 ± 0.08	0.54 ± 0.03
23	14.0 ± 0.0	0.11 ± 0.00	35.4 ± 0.6	2.5 ± 0.0	0.25 ± 0.01	1.02 ± 0.00	1.92 ± 0.03	0.53 ± 0.01
24	16.2 ± 0.6	0.13 ± 0.01	39.5 ± 0.9	2.4 ± 0.1	0.28 ± 0.01	1.10 ± 0.02	2.15 ± 0.05	0.51 ± 0.02
25	11.2 ± 0.5	0.08 ± 0.00	29.8 ± 0.9	2.7 ± 0.1	0.21 ± 0.01	0.91 ± 0.02	1.65 ± 0.04	0.55 ± 0.02
26	12.8 ± 0.9	0.10 ± 0.01	32.9 ± 0.3	2.6 ± 0.2	0.23 ± 0.01	0.97 ± 0.03	1.80 ± 0.01	0.54 ± 0.02
27	15.1 ± 0.4	0.12 ± 0.00	38.0 ± 1.8	2.5 ± 0.1	0.27 ± 0.02	1.06 ± 0.01	2.07 ± 0.10	0.51 ± 0.03
28	12.3 ± 0.3	0.09 ± 0.00	34.6 ± 0.8	2.8 ± 0.1	0.25 ± 0.01	0.95 ± 0.01	1.88 ± 0.04	0.51 ± 0.01
29	13.8 ± 0.3	0.11 ± 0.00	35.0 ± 0.7	2.5 ± 0.1	0.25 ± 0.01	1.01 ± 0.01	1.90 ± 0.04	0.53 ± 0.01
30	69.7 ± 0.1	0.69 ± 0.00	69.9 ± 0.5	1.00 ± 0.01	0.01 ± 0.02	5.53 ± 0.02	5.58 ± 0.12	0.99 ± 0.02
31	22.7 ± 1.2	0.20 ± 0.01	49.6 ± 1.8	2.2 ± 0.1	0.35 ± 0.03	1.35 ± 0.05	2.85 ± 0.15	0.47 ± 0.03
32	62.6 ± 3.4	0.61 ± 0.04	76.2 ± 1.6	1.22 ± 0.07	0.36 ± 0.11	4.3 ± 0.5	7.4 ± 0.6	0.58 ± 0.08
33	64.1 ± 2.6	0.63 ± 0.03	76.0 ± 2.4	1.19 ± 0.06	0.33 ± 0.10	4.5 ± 0.4	7.3 ± 0.8	0.62 ± 0.09
34	69.5 ± 5.7	0.68 ± 0.06	81.6 ± 2.2	1.18 ± 0.10	0.40 ± 0.21	5.5 ± 1.2	9.9 ± 1.3	0.56 ± 0.14
35	66.4 ± 3.3	0.65 ± 0.03	76.4 ± 2.3	1.15 ± 0.07	0.30 ± 0.12	4.9 ± 0.6	7.4 ± 0.8	0.66 ± 0.11
36	84.0 ± 6.7	0.83 ± 0.07	102.3 ± 2.3	1.22 ± 0.10	1.14 ± 0.66	11.5 ± 5.3		
37	19.1 ± 0.5	0.16 ± 0.01	55.7 ± 0.6	2.9 ± 0.1	0.45 ± 0.01	1.21 ± 0.02	3.42 ± 0.06	0.35 ± 0.01
38	60.0 ± 4.1	0.59 ± 0.04	75.6 ± 4.9	1.26 ± 0.12	0.39 ± 0.17	3.9 ± 0.5	7.2 ± 1.7	0.55 ± 0.15
39	39.4 ± 1.7	0.37 ± 0.02	45.1 ± 1.4	1.15 ± 0.06	0.09 ± 0.04	2.15 ± 0.10	2.51 ± 0.10	0.85 ± 0.05
40	75.1 ± 1.9	0.74 ± 0.02	81.9 ± 1.7	1.09 ± 0.04	0.27 ± 0.10	7.0 ± 0.6	10.0 ± 1.0	0.70 ± 0.09
41	39.8 ± 1.2	0.38 ± 0.01	53.0 ± 1.3	1.33 ± 0.05	0.22 ± 0.03	2.17 ± 0.07	3.15 ± 0.12	0.69 ± 0.03
42	57.3 ± 0.6	0.56 ± 0.01	68.5 ± 2.6	1.20 ± 0.05	0.26 ± 0.06	3.59 ± 0.07	5.3 ± 0.5	0.68 ± 0.07

[a] *Cf.* Table I.
[b] Samples Nos. 39 and 40: 3 h after start of repair.
[c] For mathematical reasons the width of the error band increases with increasing $A_r^{t=0}$.

of repair, have a different meaning. While Q-values represent the quotient of activities of repaired and unrepaired samples, p_{A_R}-values express the fraction of radiation damage which has been repaired.

High Q-values are obtained for the enzyme in the absence of a.r. additives or in the presence of a.r. DTT oxidized; lower Q-values were obtained, *e.g.*, by a.r. pyruvate, a.r. NaCl, and a.r. $MgCl_2$. Very low Q-values were found with the a.r. additives glyoxylate and DTT. A very high p_{A_R}-value was only calculated for a.r. CoASH ($\simeq 1$), all other a.r. additives yielded values < 0.5. Pronounced p_{A_R}-values (0.3–0.45) were obtained for the a.r. additives DTT and DTT oxidized, pyruvate, CoASAc, CoASAc + pyruvate, and malate. A very low p_{A_R}-value was derived for a.r. glyoxylate.

The non-repairable inactivation is characterized by the $D_{37}^{n.r.}$-values shown in Table III. The ratio of inactivation doses, $D_{37}^{t.}/D_{37}^{n.r.}$, is about 0.5 for most samples. A few samples show drastic deviations: a very high value (indicating a high percentage of non-repairable damage) was found, *e.g.*, for a.r. glyoxylate, and low values for a.r. DTT oxidized and a.r. pyruvate.

Post-irradiation inactivation

The p.r. inactivation behaviour of the enzyme in the absence or presence of a.r. and/or p.r. additives is characterized by $A_r^{t=30}$, $A_n^{t=30}$, τ', and p_{A_n} (*cf.* Table IV). An inspection of the τ'-values shows, that a retardation of p.r. inactivation was achieved by all a.r. and p.r. additives used in this study, most

Table IV. Post-irradiation inactivation of malate synthase in the absence or presence of p.r. additives.

Sample No.[a]	$A_r^{t=30}$ [%]	$A_n^{t=30}$ [%]	τ'[b] [h]	p_{A_n}
1	1.2 ± 0.1	34.7 ± 3.0	19.6 ± 1.6	0.00 ± 0.07
1b	2.4 ± 0.0	70.5 ± 3.0	59.5 ± 7.1	0.55 ± 0.07
1c	3.1 ± 0.3	90.7 ± 9.0	> 200	0.86 ± 0.15
1d	3.1 ± 0.1	91.0 ± 4.0	> 200	0.86 ± 0.09
19	92.3 ± 4.0	102.4 ± 5.4	> 200	1.04 ± 0.11
20	1.8 ± 0.1	51.7 ± 7.6	31.5 ± 6.5	0.26 ± 0.13
21	4.1 ± 0.1	58.3 ± 4.0	38.5 ± 4.9	0.36 ± 0.08
22	6.6 ± 0.2	59.1 ± 2.9	39.5 ± 3.5	0.37 ± 0.07
23	9.1 ± 0.1	64.9 ± 1.0	48.1 ± 1.7	0.46 ± 0.05
24	11.7 ± 0.5	72.3 ± 3.8	64 ± 10	0.58 ± 0.08
25	6.1 ± 0.2	54.6 ± 2.9	34.4 ± 2.8	0.31 ± 0.07
26	7.6 ± 0.6	59.5 ± 6.1	40.1 ± 7.9	0.38 ± 0.11
26b	9.6 ± 0.2	74.8 ± 2.0	71.6 ± 6.7	0.61 ± 0.06
27	9.2 ± 0.3	60.8 ± 2.4	41.8 ± 3.1	0.40 ± 0.06
28	7.9 ± 0.2	63.9 ± 2.1	46.4 ± 3.1	0.45 ± 0.06
29	8.7 ± 0.3	63.6 ± 2.8	45.9 ± 4.1	0.44 ± 0.07
30	68.5 ± 0.2	98.3 ± 0.3	> 200	0.97 ± 0.06
31	18.5 ± 1.1	81.7 ± 6.4	103 ± 39	0.72 ± 0.11
32	62.0 ± 1.8	99.1 ± 6.1	> 200	0.99 ± 0.11
33	57.9 ± 1.7	90.4 ± 4.5	> 200	0.85 ± 0.09
34	65.4 ± 2.3	94.2 ± 8.4	> 200	0.91 ± 0.14
35	60.8 ± 1.6	91.5 ± 5.2	> 200	0.87 ± 0.10
36	50.0 ± 1.6	59.6 ± 5.2	40.1 ± 6.5	0.38 ± 0.09
37	7.8 ± 1.1	40.8 ± 6.0	23.2 ± 3.8	0.09 ± 0.10
38	54.4 ± 2.6	90.7 ± 7.6	> 200	0.86 ± 0.13
39	30.5 ± 1.8	77.3 ± 5.6	81 ± 22	0.65 ± 0.10
40	60.2 ± 1.2	80.1 ± 2.6	94 ± 13	0.70 ± 0.07
41	29.0 ± 1.5	73.0 ± 4.4	66 ± 11	0.59 ± 0.09
42	49.3 ± 0.9	86.0 ± 1.8	138 ± 19	0.79 ± 0.07

[a] *Cf.* Tables I and II.

[b] For mathematical reasons the width of the error band increases drastically with increasing τ'. Values of $\tau' > 200$ h indicate more or less the absence of p.r. inactivation.

pronounced with the a.r. additives DTT, glyoxylate, malate, and CoASAc + pyruvate, and the p.r. additives $MgCl_2$ and glyoxylate (in the absence of a.r. $MgCl_2$), and least pronounced with a.r. pyruvate and a.r. DTT oxidized. Similar conclusions concerning p.r. inactivation may be drawn from the values for the normalized protective efficiency, p_{A_n}.

Correlation of protection and repair-promotion efficiencies of a.r. additives

Two-dimensional correlation plots of the normalized parameters, p_{A_r}, p_{A_n}, and p_{A_R}, have been drawn. For completeness, some normalized parameters have been calculated from residual activities given in a previous paper [8] and have been included in these plots.

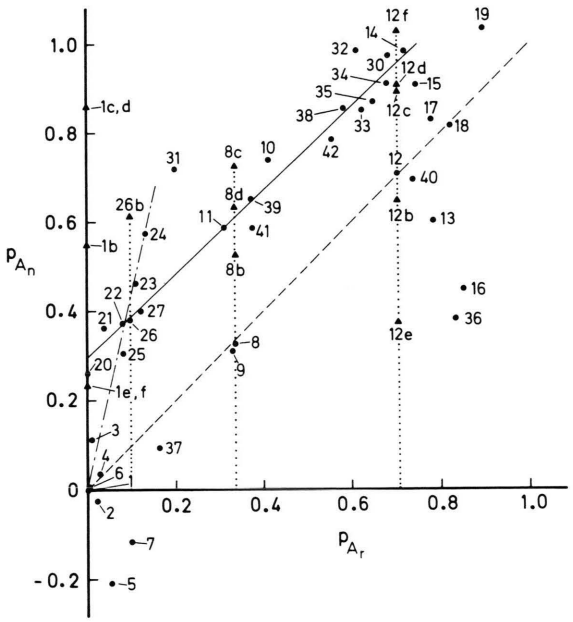

Fig. 1. Correlation of protective efficiency against p.r. inactivation, p_{A_n}, and of protective efficiency against primary inactivation, p_{A_r}. ●: a.r. additives, numbering follows the designation of samples given in Table I; ▲: p.r. additives, numbering is the same as in Table II; ·····: auxiliary lines for p.r. additives; -----: regression line for samples Nos. 1, 8, 12 (0, 10, 100 mM a.r. formate), coinciding with the median line ("isoprotective line"); -·-·-: regression line for samples Nos. 1, 22–24 (0, 100, 200, 400 mM a.r. NaCl); ——: regression line (r = 0.9806) for samples Nos. 21, 22, 26, 30, 32–35, 38, 39, 41, 42 (100 mM of a.r. additives).

Clear correlations can only be recognized in a plot combining primary and p.r. inactivation (Fig. 1). This may be seen, *e.g.*, from the data plotted for different concentrations of a.r. formate or a.r. NaCl, or for the same concentration (100 mM) of most a.r. additives (*cf.* the regression lines shown in the figure). The plot in Fig. 1 also shows that, with the exception of a few examples, the protective efficiency of a.r. additives against post-irradiation inactivation exceeds the efficiency against primary inactivation. The inactivation-stimulating effect of a.r. SOD (especially in high concentration) in the p.r. phase (*cf.* [8]) is reflected, *e.g.*, by the points below the median line, especially by the negative p_{A_n}-values.

No clear correlations could be derived from two-dimensional plots combining p_{A_R} with p_{A_r} or p_{A_n} (plots not shown).

Statements concerning correlations between primary inactivation, p.r. inactivation, and extent of repair may be drawn from the ternary diagram shown in Fig. 2. The majority of points cluster in two definite areas of the diagram. The smaller cluster comprises the inorganic salts and CoASAc, the larger one most of the other a.r. additives. A few a.r. additives are clearly outside these areas: pyruvate, CoASH, Tris/HCl, DTT oxidized, cata-

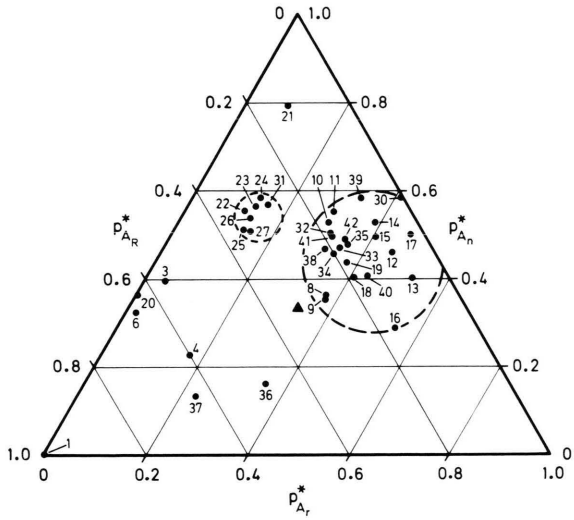

Fig. 2. Ternary diagram, correlating renormalized efficiencies $p^*_{A_r}$, $p^*_{A_n}$, and $p^*_{A_R}$. Numbering of samples is given in Table I. Most a.r. additives are gathering in two clusters (indicated by circles). The "isoeffective point" ($p^*_{A_r}$ = $p^*_{A_n}$ = $p^*_{A_R}$ = 1/3) is marked by ▲.

lase, SOD + catalase, and, per definitionem, the enzyme without a.r. additives. It should be noted, that three further samples containing a.r. SOD (Nos. 2, 5, and 7), which yielded negative p_{A_n}-values, cannot be included in this diagram, because the corresponding points would lie outside.

Comparison of protective efficiencies for samples with p.r. additives

In some cases p.r. additives were added to samples irradiated in the absence or presence of a.r. additives (*cf.* Table IV). The results from these experiments (together with some data adopted from [8]) have been included in Fig. 1, in order to compare p_{A_n} (obtained with p.r. additives), and p_{A_r} (obtained without p.r. additives). The results unveil a protective efficiency against p.r. inactivation provided by p.r. additives (glyoxylate, $MgCl_2$, NaCl, formate, catalase, SOD at low concentration of a.r. formate). An enhancement of p.r. inactivation by p.r. SOD is observed in the presence of a high concentration of a.r. formate.

Discussion and Conclusions

The main aim of this study was the characterization of a large number of additives with respect to their ability to modulate primary inactivation, post-irradiation inactivation, and repair of X-irradiated malate synthase. This was accomplished by screening experiments on the microlevel. To perform a rapid screening, conditions had to be accepted which are not optimum from the point of view of radiation chemistry: *e.g.*, single-dose experiments, a possible depletion of oxygen during irradiation, only one sampling time in the p.r. phase (*cf.* Materials and Methods).

Despite the mentioned shortcomings, useful comparisons of the various additives can be achieved. For this purpose the normalized efficiency parameters, p_{A_r}, p_{A_n}, p_{A_R}, were established which reflect the three effects under investigation. Binary diagrams (*e.g.*, Fig. 1) were used to correlate two out of the three mentioned parameters. Additional information was derived from a ternary diagram, constructed from renormalized parameters, $p^*_{A_r}$, $p^*_{A_n}$, $p^*_{A_R}$ (Fig. 2). As a consequence of the normalization to $\Sigma p^*_{A_i} = 1$, these quantities describe the effects on a relative scale (giving information on the fractions of the respective effects). The two different kinds of presentation are complementary.

Most of the a.r. additives investigated turned out to protect the enzyme against primary and p.r. inactivation and to enhance the reparability of enzymic activity by DTT. A comparison of radioprotective effects of various a.r. additives against damages of enzyme structure (subunit cross-linking, enzyme aggregation, fragmentation, unfolding) has already been established on the basis of results obtained from electrophoreses and small-angle X-ray scattering experiments [13].

In the present study the protective efficiency of a.r. additives against p.r. inactivation generally was superior to that against primary inactivation.

Statements concerning differences in the efficiencies of some a.r. additives may be deduced from Figs. 1 and 2: There is a considerable difference between the effects of glyoxylate and pyruvate, or between CoASH and CoASAc, or between CoASAc and pyruvate on one hand and CoASAc + pyruvate on the other, or between reduced and oxidized forms of DTT. No significant difference was found for the different malates, (L), (D), (DL), (L) + (D). Differing effects of a.r. additives (*e.g.*, glyoxylate, pyruvate, CoASAc, malate) on the X-ray induced aggregation of malate synthase have already been established by small-angle X-ray studies [5, 10].

The substantially different effects of the a.r. additives CoASH and CoASAc, or of DTT and DTT oxidized may, at least partly, be explained by the thiol character of CoASH and DTT, and the lack of free sulfhydryls in CoASAc and DTT oxidized.

The differences in the behaviour of the a.r. additives glyoxylate and pyruvate are puzzling: the efficiency of glyoxylate clearly exceeds that of pyruvate for primary and p.r. inactivation, but glyoxylate is much less effective than pyruvate when considering the promotion of DTT-repair and the previously investigated X-ray induced aggregation of malate synthase [5, 10]. The differences in space-filling and in binding constants between the substrate glyoxylate and the analogue pyruvate may serve as possible explanation for this behaviour.

The differences between the a.r. additives CoASAc and pyruvate and the combination CoASAc + pyruvate are presumably due to different conformational states of the enzyme (and thereby of its active site) (*cf.* [21–23]).

As follows from an inspection of Fig. 2, a.r. glyoxylate exhibits an excellent protective effect against both sorts of inactivation, and no significant repair promotion. This is obviously due to a maximum protection of the essential sulfhydryls of the enzyme by the substrate glyoxylate. It should be emphasized that p.r. glyoxylate may also act as potent protective against p.r. inactivation (*cf.* Fig. 1).

The enhancement of p.r. inactivation by p.r. SOD in the presence of a high concentration of a.r. formate may be due to the increased formation of H_2O_2 as a consequence of secondary reactions. This explanation is supported by the findings for p.r. SOD + catalase (*cf., e.g.,* sample No. 12d with 12b in Fig. 1).

There is a clear difference between the protective efficiencies of a.r. and p.r. catalase against p.r. inactivation (*cf.* Fig. 1): a.r. catalase was more protective in low concentration (*cf.* sample No. 3 with 6, and No. 14 with 17), p.r. catalase in high concentration (*cf.* sample No. 12f with 12c). A plausible explanation for this different behaviour may be the action of iron ions released from X-ray damaged a.r. catalase (*cf.* [1, 2, 28]).

Some of the effects found for substrates, products, and analogues, may be attributed to a specific protection of the enzyme. Such specific protective effects may comprise (i) shielding of cysteine or other sensitive amino acids in the catalytic site or its near surroundings by the ligands themselves, (ii) ligand-induced changes of the tertiary structure leading to a more radiation-resistant enzyme conformation (*e.g.,* reduced exposition of sensitive amino acids on the enzyme surface), (iii) ligand-induced changes of the enzyme environment (*i.e.,* changes of hydration and of preferential ligand binding). All mentioned structural effects may finally influence the radiation resistance of the enzyme. Since the specific ligands were present in solution in excess (to guarantee sufficient binding), they may have acted additionally through radical scavenging. This may be concluded from the known rate constants $k_{\cdot OH}$ (in M^{-1} s^{-1}) [29]: 3.1×10^7 for pyruvate (pH 9), 7.1×10^8 for glycollate (pH 9), 8.6×10^8 for malate, and 4.8×10^9 for the unspecific ligand lactate (pH 9). Both a specific protection by substrates or coenzymes and protection through radical scavenging have been reported in the case of other enzymes (*cf., e.g.* [30–33] and [34], respectively).

The action of sulfhydryl agents also represents some kind of specific protection: they protect the enzyme sulfhydryls, located in the active site region or anywhere. Sulfhydryl compounds like DTT are capable to maintain and/or restore the integrity of enzyme sulfhydryls. Furthermore, in addition to their reducing power they may additionally act effectively by radical scavenging (*cf.* [35]). It was already demonstrated [36], that sulfhydryl agents may protect enzymes which are void of both sulfhydryls and disulfides.

The present study has shown how a variety of additives can be compared with respect to their efficiency to protect an irradiated enzyme and to promote its repair. Such screening experiments, however, will have to be supplemented by more detailed investigations of selected additives (*e.g.,* under definite oxic or anoxic conditions, under variation of enzyme and/or additive concentration, irradiation dose, sampling time in the p.r. phase), to obtain more information on the detailed mechanisms of enzyme inactivation and protection.

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