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POST-IRRADIATION INACTIVATION OF THE SULFHYDRYL ENZYME MALATE SYNTHASE*

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SUMMARY. The sulfhydryl enzyme malate synthase was inactivated in air-saturated aqueous solution by X-irradiation (2 kGy). Changes of activity were registered up to 60 h after irradiation. Effects of specific additives (formate, superoxide dismutase, catalase), added before and/or after irradiation, revealed the role of the deleterious radical and non-radical species responsible for the radiation damage: inactivation during irradiation is mainly due to the action of $OH^{\cdot}$, to a minor extent to $O_2^{\cdot-}$ and H_2O_2; post-irradiation inactivation is mainly caused by H_2O_2. A partial restoration of enzyme activity by dithiothreitol, added after irradiation, was found for all systems investigated; repairs were significant even when they were initiated 60 h after irradiation.

Malate synthase, the second key enzyme of the glyoxylate cycle, catalyzes the Mg^{2+} dependent synthesis of malate from acetyl-CoA and glyoxylate. The enzyme from baker's yeast has been isolated in an electrophoretically pure form (1) and has been characterized by various physico-chemical techniques (2-6). The enzyme was reported to have a molecular weight of about 185000 (4,1); the quaternary structure was suggested to be trimeric (6,1); the available sulfhydryls (1 per subunit) were found to be essential for enzymic activity (6,7).

Extensive X-irradiation of aqueous solutions of malate synthase resulted in pronounced damages of the enzyme. The investigation of these damages by various techniques (small-angle X-ray scattering, electrophoreses, sulfhydryl determinations, enzymic tests) demonstrated that the enzyme suffers aggregation, fragmentation, partial unfolding, inactivation, and a loss of sulfhydryls (3,7-11). Enzyme sulfhydryls were found to play an essential role in inactivation and aggregation phenomena (7).

* Dedicated to Professor Dr. Erwin Schauenstein on the occasion of his 65th birthday.

Abbreviations and Enzymes: *a.r.*, *ante radiationem*; *p.r.*, *post radiationem*; 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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The presence of DTT or of substrates or analogues during X-irradiation protected malate synthase against aggregation, subunit cross-linking, and inactivation, but not against fragmentation (7,8). Post-irradiation treatment of the enzyme solutions with DTT led to a significant repair of enzymic activity; the repair phenomenon was ascribed to the restoration of the essential sulfhydryls (7).

Under conditions of our experiments (air-saturated aqueous solutions) mainly $OH^{\cdot}$ and $O_2^{\cdot-}$ radicals and H_2O_2 were held responsible for the observed radiation damages of malate synthase (7,8). To get further insight into the role of these deleterious species during X-irradiation and in the post-irradiation phase, inactivation, protection and repair experiments were performed in the presence of a variety of appropriate additives: formate as $OH^{\cdot}$ scavenger, SOD as $O_2^{\cdot-}$ scavenger, catalase as a scavenger for H_2O_2.**

MATERIALS AND METHODS

Materials. DTT was obtained from Serva, Heidelberg; sodium formate was from Merck, Darmstadt; catalase from bovine liver and SOD from bovine erythrocytes were purchased from Boehringer, Mannheim. All other reagents were of A-grade purity. Quartz-bidistilled water was used throughout. Malate synthase was isolated from baker's yeast as described elsewhere (1); the specific activities of different preparations amounted to 40-50 IU/mg.

Solutions. 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 as a standard buffer. Malate synthase was dialyzed and stored at 2°C using this buffer. Concentrations of enzymes and additives were determined spectrophotometrically or gravimetrically. For irradiation experiments stock solutions of malate synthase and *a.r.* additives were mixed to give the following final concentrations: 2.7 µM (= 0.5 mg/ml) malate synthase, 0 or 10 or 100 mM formate, 0 or 0.4 or 4 µM SOD, 0 or 55 or 550 nM catalase.

X-Irradiation. The X-ray source was a tube with copper target (Philips PW 2253/11) that was operated at 50 kV. Solutions were irradiated in the microcell described elsewhere (7,8); the cell volume was adjusted to 240 µl. Samples were irradiated at 2°C with 2 kGy (dose rate 160 Gy/min) and stored afterwards at about the same temperature.

Enzymic Assay. The assay was performed at 20°C as described previously (12,1). Aliquots of the mixtures (1-20 µl) were used in the enzymic test as outlined in (7). Each test was performed at least 3 times; accuracy of activities was better than 5 %. The irradiated solutions were tested at about 0, 30, 60 h after stop of irradiation. The activity of the unirradiated references turned out to be almost unaffected by the presence of additives. The ratio of the activities of irradiated and unirradiated samples will be called residual activity A_r.

Post-Irradiation Repair. Repair experiments were carried out by addition of concentrated DTT solution to yield a final concentration of 10 mM. DTT was added to the irradiated mixtures at about 0, 30, 60 h after stop of irradiation. Activity tests were performed 3 and 30 h after addition of DTT.

Post-Irradiation Treatment. In order to elucidate the influence of *p.r.* additives, to aliquots of some irradiated samples solutions of formate or SOD and/or catalase were added after stop of irradiation. Enzyme activities were tested 30 h after addition of *p.r.* additives.

RESULTS AND DISCUSSION

X-irradiation experiments with malate synthase were performed with 20 different reaction mixtures, varying the concentration of *a.r.* additives formate, SOD and

** Some of the results were presented at the 7th Int. Congr. of Radiation Research, Amsterdam 1983, Abstr. No. A4-09.

Table 1

Samples	*a.r.* Additives			A_r at t ≅ 0 (%)	*p.r.* Additives			k' (h^{-1}) x 10^2	q-Values
	Formate (mM)	SOD (μM)	Catalase (nM)		Formate (mM)	SOD (μM)	Catalase (nM)		
1/5/9	0/10/100	0	0	3.2/35.0/69.5	0	0	0	3.75/1.83/0.79	1.00/1.00/1.00
					0	0.36	0	3.18/1.28/0.90	0.85/0.70/1.14
					0	0	50	3.35/0.62/0.14	0.89/0.34/0.18
					0	0.33	45	2.20/0.97/0.23	0.59/0.53/0.29
2/6/10	0/10/100	0.4	0	5.2/35.3/77.2	0	0	0	4.47/1.98/1.01	1.00/1.00/1.00
					0	0	50	3.71/0.88/0.24	0.83/0.44/0.24
3/7/11	0/10/100	0	55	3.7/42.9/72.1	0	0	0	2.67/0.73/0.08	1.00/1.00/1.00
					0	0.36	0	2.92/0.89/0.33	1.09/1.22/4.13
					0	0	50	1.95/0.68/0.07	0.73/0.93/0.88
					0	0.33	45	2.63/0.91/0.35	0.99/1.25/4.38
4/8/12	0/10/100	0.4	55	5.3/33.8/73.9	0	0	0	3.20/1.27/0.22	1.00/1.00/1.00
					0	0	50	3.00/1.27/0.10	0.94/1.00/0.45

Composition of samples 1-12, residual activities at time t ≅ 0 after 2 kGy X-irradiation, and *p.r.* inactivation in the absence/presence of *p.r.* additives. A_r, residual activity (in % of the activity of the unirradiated sample); k', apparent first-order rate constant of *p.r.* inactivation, obtained from A_r-values at t ≅ 0 and t ≅ 30 h *p.r.*; q, quotient of k'-values in the presence and absence of a definite *p.r.* additive.

Table 2

Samples	*a.r.* Additives			A_r at $t \cong 0$ (%)	*p.r.* Additives			k' (h^{-1}) x 10^2	q-Values
	Formate (mM)	SOD (μM)	Catalase (nM)		Formate (mM)	SOD (μM)	Catalase (nM)		
13/17	0/100	0	0	3.6/74.7	0	0	0	3.75/0.70	1.00/1.00
					90	0	0	2.55/ -	0.68/ -
					0	3.6	0	3.35/1.92	0.89/2.74
					0	0	500	2.96/0.09	0.79/0.13
14/18	0/100	4.0	0	8.5/85.6	0	0	0	5.00/1.46	1.00/1.00
					90	0	0	3.41/ -	0.68/ -
					0	3.6	0	4.69/2.11	0.94/1.45
					0	0	500	4.27/0.83	0.85/0.57
15/19	0/100	0	550	3.8/79.0	0	0	0	2.81/0.36	1.00/1.00
					90	0	0	1.44/ -	0.51/ -
					0	3.6	0	2.39/0.35	0.85/0.97
					0	0	500	2.56/0.41	0.91/1.14
16/20	0/100	4.0	550	13.9/83.0	0	0	0	5.53/0.39	1.00/1.00
					90	0	0	2.19/ -	0.40/ -
					0	3.6	0	3.48/0.38	0.63/0.97
					0	0	500	3.63/0.40	0.66/1.03

Composition of samples 13-20, residual activities at time $t \cong 0$ after 2 kGy X-irradiation, and *p.r.* inactivation in the absence/presence of *p.r.* additives. A_r, residual activity (in % of the activity of the unirradiated sample); k', apparent first-order rate constant of *p.r.* inactivation, obtained from A_r-values at $t \cong 0$ and $t \cong 30$ h *p.r.*; q, quotient of k'-values in the presence and absence of a definite *p.r.* additive.

catalase. The composition of the samples used for X-irradiation is listed in Tables 1 and 2.

Immediately after X-irradiation aliquots of the irradiated samples were tested, treated by *p.r.* addition of formate or SOD and/or catalase, or repaired by DTT.

Primary Inactivation

The residual activities of the irradiated untreated samples, measured shortly after X-irradiation, are documented in Tables 1 and 2. The presence of OH' scavenging formate during X-irradiation obviously exhibits a strong protective effect. The extent of this effect is more pronounced at the higher formate concentration used. The simultaneous presence of *a.r.* SOD and/or *a.r.* catalase enhances this protection generally. The effect of SOD and catalase depends both on the concentration of these *a.r.* additives and that of *a.r.* formate. At a given *a.r.* formate concentration the *a.r.* additives SOD and/or catalase are more potent protectors at high concentrations than at low concentrations.

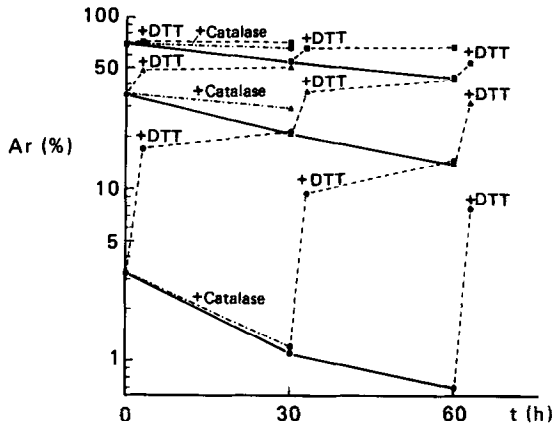

Fig.1: Residual activity A_r of malate synthase as a function of storage time t after X-irradiation. 2 kGy irradiation in the absence (●) or presence of 10 mM (▲) or 100 mM (■) formate. ——: post-irradiation inactivation; _ _ _: repair by DTT; _.._.: inhibition of inactivation by catalase added after irradiation.

Post-Irradiation Inactivation

The residual activities of the irradiated untreated samples, measured 30 and 60 h *p.r.*, show a decay with increasing storage time. A few examples (samples 1, 5, 9 of Table 1) are illustrated in Fig.1. The figure clearly shows that the percentual decay is more pronounced the lower the residual activity after stop of irradiation. In order to compare the influence of *a.r.* additives on the *p.r.* inactivation we formally calculated apparent first-order rate constants k' from the residual activities at 0 and 30 h *p.r.* (*cf.* Tables 1 and 2: values in the absence of *p.r.* additives). The *a.r.* additives SOD and/or catalase yield the following general order of k'-values: SOD > none > SOD+catalase > catalase; this series holds at 0, 10, 100 mM formate, with the exception of sample 16. The k'-values obtained at high *a.r.* concentrations of SOD and/or catalase turn out to be higher than the values at low *a.r.* concentrations of these additives (*cf.* Tables 1 and 2).

The addition of *p.r.* additives influences the *p.r.* inactivation considerably (*cf.* Tables 1 and 2). In order to facilitate the comparison between the effects caused by the different *p.r.* additives, we adopted the following procedure: For each irradiated sample we calculated the quotients q of the k'-values in the presence of the various *p.r.* additives and the corresponding k'-value in the absence of *p.r.* additives. An inspection of the quotients summarized in Tables 1 and 2 allows the following statements:

(i) The *p.r.* addition of catalase reduces the *p.r.* inactivation significantly (Tables 1 and 2: $q < 1$; *cf.* also Fig.1). The effect is generally less pronounced or absent with the samples where catalase or SOD+catalase have been added before irradiation.

(ii) The *p.r.* addition of SOD causes different effects ($q \lesssim 1$), depending on the presence and concentration of *a.r.* additives. A protective effect of *p.r.* SOD

is only exhibited in the absence of high *a.r.* formate concentration. In some cases a considerably enhanced *p.r.* inactivation is observed at high *a.r.* formate concentration.

(iii) In the absence of *a.r.* SOD and *a.r.* catalase the *p.r.* addition of SOD+ catalase inhibits the *p.r.* inactivation greatly. In the presence of *a.r.* catalase the effects of *p.r.* SOD+catalase are similar to those of *p.r.* SOD.

(iv) In the presence of *p.r.* formate a protective effect is found which is more pronounced in the presence of *a.r.* catalase or *a.r.* SOD+catalase.

Repair

Repair experiments by means of DTT (performed 0, 30, 60 h after irradiation) established for all 20 samples that both the primary inactivation as well as the *p.r.* inactivation can be compensated to a high degree by DTT. The activity which can be regained by DTT decreases with increasing storage time, *i.e.* with increasing inactivation. The percentual repair is more pronounced in the absence of *a.r.* formate, as is illustrated for the examples presented in Fig.1. In general, in the presence of *p.r.* additives similar repair phenomena by DTT are observed as in the absence of *p.r.* additives.

CONCLUSIONS

The pronounced radioprotective effect of *a.r.* formate clearly indicates that the $OH^{\cdot}$ radical is mainly responsible for the damage of malate synthase during X-irradiation. This result is in accord with the findings on other systems in air-saturated aqueous solution (*cf.* 13,14). The results in the presence of *a.r.* SOD and/or *a.r.* catalase demonstrate that obviously also $O_2^{\cdot-}$ and H_2O_2 are involved to a minor extent in the primary inactivation of malate synthase. The ability of $O_2^{\cdot-}$ radicals and of H_2O_2 to inactivate sulfhydryl enzymes is also known from other studies (*cf.* 13-15).

The observed series of *a.r.* additives for the efficiency against *p.r.* inactivation allows clear statements about the role of H_2O_2 in the post-irradiation inactivation: Both the pronounced protection of *a.r.* catalase against *p.r.* inactivation and the enhancement of *p.r.* inactivation by *a.r.* SOD (*via* formation of H_2O_2) establish that H_2O_2 is the predominant inactivating species in the *p.r.* phase. The effects of *p.r.* added catalase corroborate this finding too.

The effects caused by *p.r.* SOD indicate an involvement of $O_2^{\cdot-}$ in the post-irradiation phase. This conclusion may be deduced from the observed protection by *p.r.* SOD at low *a.r.* formate concentrations as well as from the enhanced *p.r.* inactivation in the presence of high *a.r.* formate. By means of secondary reactions $O_2^{\cdot-}$ radicals may be generated from various sources (*cf.* 14). The protective effect of *p.r.* formate suggests the occurrence of $OH^{\cdot}$ radicals also in the *p.r.* phase.

The restoration of the activity of X-irradiated malate synthase by means of DTT is obviously due to a restoration of essential enzyme sulfhydryls, possibly by

reduction of disulfides and/or sulfenic acid products. The formation of sulfenic acid products may be ascribed to the action of H_2O_2 (*cf.* 16,17).

It can be expected that the results found for the sulfhydryl enzyme malate synthase are also valid for other enzymes with essential sulfhydryls. Detailed investigations on repair phenomena of malate synthase in the presence of *a.r.* and *p.r.* additives will be reported in a subsequent paper.

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