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Small-Angle X-Ray Scattering Studies on the X-Ray Induced Aggregation of Malate Synthase II. Inactivation and Aggregation Experiments

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The X-ray induced inactivation and aggregation of the enzyme malate synthase in aqueous solution were investigated by small-angle X-ray scattering and enzymic tests.

Enzymic activity decreases about exponentially with increasing dose. The rate of inactivation depends linearly on the dose rate; the extrapolation to zero dose rate yielded a finite limiting value of the rate constant of inactivation. The inactivation dose D_{37} is a linear function of enzyme concentration.

Enzymic tests and small-angle X-ray scattering on samples, which had been X-irradiated before the measurements, showed no direct relation between aggregation and inactivation. The substrates glyoxylate and acetyl-CoA and the substrate analogue pyruvate are able to protect the enzyme against radiation damage, however to a different extent against aggregation (pyruvate > glyoxylate > acetyl-CoA) or inactivation (glyoxylate > pyruvate ≫ acetyl-CoA; the latter showed no effect). These findings and the protective effect of dithiothreitol against aggregation and inactivation of the enzyme are discussed in context with the formation of hydrogen peroxide. A possible shielding of radiosensitive groups of the enzyme by the substrates and scavenging are also taken into consideration as explanations for the protective effects.

While the novel application of small-angle X-ray scattering in the field of radiation biology delivers information on X-ray induced structural changes of biopolymers and on their kinetics, the occurrence of radiation damages in conventional small-angle X-ray scattering measurements on biopolymers can be reduced by a variety of precautions.

(Keywords: Aggregation; Inactivation; Malate Synthase; Radioprotection; Small-Angle X-Ray Scattering; X-Ray Damage)

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Röntgenkleinwinkeluntersuchungen der durch Röntgenstrahlen induzierten Aggregation der Malatsynthase. II. Inaktivierungs- und Aggregationsexperimente

Die durch Röntgenbestrahlung in wäßriger Lösung induzierte Inaktivierung und Aggregation des Enzyms Malatsynthase wurden mit der Methode der Röntgenkleinwinkelstreuung und mit Enzymtests untersucht.

Die Enzymaktivität nimmt mit zunehmender Dosis annähernd exponentiell ab. Die Inaktivierungsgeschwindigkeit hängt linear von der Dosisleistung ab; die Extrapolation auf Dosisleistung null ergab einen endlichen Grenzwert der Inaktivierungsgeschwindigkeitskonstante. Die Inaktivierungsdosis D_{37} ist eine lineare Funktion der Enzymkonzentration.

Enzymtests und Röntgenkleinwinkelstreuexperimente an Proben, welche vorher bereits bestrahlt worden waren, zeigten keinen direkten Zusammenhang zwischen Aggregation und Inaktivierung. Die Substrate Glyoxylat und Acetyl-CoA sowie das Substratanaloge Pyruvat vermögen das Enzym gegen Strahlenschäden zu schützen, jedoch in unterschiedlichem Ausmaß gegen Aggregation (Pyruvat > Glyoxylat > Acetyl-CoA) oder Inaktivierung (Glyoxylat > Pyruvat $\gg$ Acetyl-CoA; letzteres zeigte keinen Effekt). Diese Befunde und die Schutzwirkung von Dithiothreitol gegen die Aggregation und Inaktivierung des Enzyms werden in Zusammenhang mit der Bildung von Wasserstoffperoxid diskutiert. Als weitere Erklärungen für die Schutzwirkungen werden die mögliche Abschirmung strahlenempfindlicher Gruppen des Enzyms durch die Substrate und der Einfang von Radikalen in Erwägung gezogen.

Während die neuartige Anwendung der Röntgenkleinwinkelstreuung auf dem Gebiet der Radiobiologie Aufschluß über strahleninduzierte Strukturänderungen von Biopolymeren und deren Kinetik gibt, läßt sich das Auftreten von Strahlenschäden bei konventionellen Röntgenkleinwinkelmessungen an Biopolymeren durch verschiedene Vorkehrungen reduzieren.

Introduction

In the preceding paper[1] we have shown the occurrence of an X-ray induced aggregation of malate synthase upon irradiation of aqueous solutions of the enzyme. The formation of aggregates was monitored in situ by the small-angle X-ray scattering (SAXS) technique. Based on the experimental SAXS results a model for the aggregation process was developed[1,2]. According to this model, which can be supported by computer simulations[3], the aggregation process is dominated by a two-dimensional aggregation. The binding sites for cross-linking were assumed to be situated on the periphery of the oblate enzyme particles.

The technique of SAXS can only register changes of the overall structure of the enzyme upon X-irradiation. No statements can be made by this technique on changes of the enzymic function upon X-irradiation. Therefore, enzymic tests have to be carried out to gain information on the dependence of enzymic activity on the radiation dose. Moreover, in order to establish a structure-function relationship of radiation damaged particles, measurements have to include structural and functional investigations on the same enzyme sample. That means,

SAXS and enzymic tests must be performed on samples which have been irradiated with a defined X-ray dose prior to the scattering and activity measurements. The present paper reports on these investigations.

Materials and Methods

Materials, enzyme solutions, X-ray source, SAXS measurements were as described in Part I[1], except for the following conditions: The enzyme concentration of the stock solution amounted to about 14 mg/ml; solutions of lower concentration as used for the inactivation experiments were prepared by diluting the stock solution with dialysis buffer. The final concentrations of the ligands for the inactivation experiments amounted to 5 or 50 *mM* glyoxylate, 5 or 50 *mM* pyruvate, 2 *mM* acetyl-CoA; and amounted to 50 *mM* glyoxylate, 50 *mM* pyruvate, 3 *mM* acetyl-CoA for the scattering experiments. This corresponds to a degree of saturation of the enzyme to about 90-99%. The current for the X-ray tube was 30 mA except for some experiments when it was varied from 4 to 30 mA. In the SAXS measurements, only the data from the first run were used for the final evaluation.

Irradiation Experiments

X-irradiation of the enzyme and of enzyme-substrate complexes was performed by means of two types of cells described elsewhere[2,4]. The large cell (1.25 ml volume) was only used for some experiments with low-concentrated solutions at room temperature, the small cell (70 µl volume), which could be thermostated to a temperature between 2 and 10 °C, for all other experiments. In both cells the samples were irradiated by the unfiltered radiation. X-ray dosimetry* was performed by means of a Fricke dosimeter as described previously[2].

Enzymic Assay

For the enzymic assay the rate of cleavage of the thioester bond of acetyl-CoA was measured directly at 232 nm[5]. The test was performed in a 0.5 cm quartz cuvette in pyrophosphate buffer[6,7] at 20 °C in a Zeiss PMQ II spectrophotometer. Enzyme concentrations in the cuvette amounted to 0.5-5 µg/ml. The specific activities found were independent of concentration; they varied from about 15-30 U/mg for different samples of the unirradiated enzyme. Irradiated solutions were tested within a few minutes after the end of X-irradiation.

Determination of Hydrogen Peroxide

The amount of hydrogen peroxide in irradiated solutions was estimated spectrophotometrically at 410 nm using the well-known reaction with $TiOSO_4$. The reliability of the determination was checked by means of a solution of known hydrogen peroxide content.

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* The absorbed dose is given in rd, while rad is used as unit for the scattering angle 2ϑ.

Results

1. Inactivation Experiments

The Influence of Dithiothreitol (*DTT*)

Tests of enzymic activity after X-irradiation of the substrate-free enzyme showed that the activity decreases about exponentially with increasing time of irradiation. As can be seen from the results shown in Fig. 1a (curve 1), the data obtained from measurements on two enzyme solutions which contained 0.2 and 2 *mM DTT*, respectively, can be fitted well by the same straight line, thus yielding the same value for the first-order rate constant of inactivation k_i. This finding suggests that the concentration of *DTT* may not have an influence on k_i. On the other hand, since the enzyme solutions used for these experiments had been stored for some time in the presence of *DTT*, *DTT* might well have suffered autoxidation during this time.

Therefore we set up another series of experiments and added freshly dissolved *DTT* to an enzyme solution that had been stored for a long time in the presence of 0.2 *mM DTT*. The results from these experiments are shown in Fig. 1b. As the figure convincingly demonstrates, fresh *DTT* has a remarkable influence on the rate of inactivation. Upon raising the concentration of *DTT* from 0.2 *mM* to 0.7 *mM* by adding fresh *DTT*, k_i decreases to about 16% of its original value, and after increasing the concentration of *DTT* to 2.2 *mM*, k_i decreases further to about 11% of its original value. These findings correspond to an increase of the inactivation dose D_{37} of the enzyme from 39 krd (before the addition of fresh *DTT*) to values of 244 krd and 346 krd (after the addition of fresh *DTT*).

Obviously this protection against radiation damage viz. inactivation is provided only by *DTT* in its reduced form, freshly dissolved, and not by *DTT* that is already oxidized. This behaviour seems to be in contrast to the previously described influence of *DTT* on the rate of aggregation of the enzyme[1] (Part I). Although many of our previous SAXS experiments were performed also with enzyme solutions that had been stored in the presence of *DTT* for some time, we always observed a remarkable difference in the inhibition of X-ray induced aggregation between 0.2 and 2 *mM DTT*.

The Influence of Substrates or Analogues

Fig. 1a presents also results obtained with various enzyme-substrate complexes. It shows that the presence of 2 *mM* acetyl-CoA does not change the rate of inactivation. A similar concentration of acetyl-CoA (corresponding to a 95% saturation of the enzyme) turned out to be able to reduce the rate of X-ray induced aggregation (cf. Part I).

The presence of 50 *mM* glyoxylate ($\approx$ 100% saturation) causes a decrease of k_i to about 17% of the value for the substrate-free enzyme. Surprisingly enough, the data obtained in the presence of 5 *mM* glyoxylate are so similar to those obtained in the presence of 50 *mM* pyruvate ($\approx$ 98-99% saturation for both ligands), that they can be

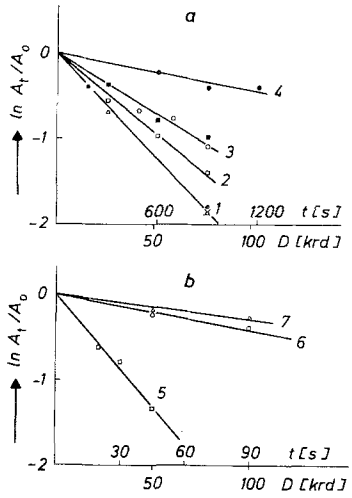

Fig. 1. Logarithmic plots of the residual activity A_t/A_0 of malate synthase vs. the time t of X-irradiation as a function of the concentration of *DTT* and of the substrates. *a* Irradiation in the large cell (dose rate $D/t \approx 86$ rd/s, enzyme concentration $c \approx 0.075$ mg/ml). 1: (Δ) 0.2 *mM DTT*, (▲) 2 *mM DTT*, (×) 2 *mM* acetyl-CoA + 0.2 *mM DTT*; rate constant of inactivation $k_i = 2.1 \cdot 10^{-3}\,s^{-1}$, $D_{37} = 41$ krd; 2: (□) 5 *mM* pyruvate + 0.2 *mM DTT*, $k_i = 1.6 \cdot 10^{-3}\,s^{-1}$, $D_{37} = 54$ krd; 3: (■) 50 *mM* pyruvate + 0.2 *mM DTT*, (○) 5 *mM* glyoxylate + 0.2 *mM DTT*; $k_i = 1.2 \cdot 10^{-3}\,s^{-1}$, $D_{37} = 72$ krd; 4: (●) 50 *mM* glyoxylate + 0.2 *mM DTT*, $k_i = 3.6 \cdot 10^{-4}\,s^{-1}$, $D_{37} = 240$ krd. *b* Irradiation in the small cell ($D/t \approx 1.1$ krd/s, $c \approx 0.38$ mg/ml, 0.2 *mM DTT*). 5: (□) no further *DTT* added, $k_i = 2.8 \cdot 10^{-2}\,s^{-1}$, $D_{37} = 39$ krd; 6: (○) 0.5 *mM* fresh *DTT* added, $k_i = 4.6 \cdot 10^{-3}\,s^{-1}$, $D_{37} = 244$ krd; 7: (Δ) 2 *mM* fresh *DTT* added, $k_i = 3.3 \cdot 10^{-3}\,s^{-1}$, $D_{37} = 346$ krd

approximated in the logarithmic plot by the same straight line. The corresponding rate constant is by a factor of about 3 larger than that determined in the presence of 50 *mM* glyoxylate. This shows that pyruvate is a much poorer inhibitor of the inactivation than glyoxylate. The rate constant of inactivation in the presence of 5 *mM* pyruvate ($\approx$ 90% saturation) is by about 30% larger than at 50 *mM* pyruvate, but still by about the same amount smaller than k_i for the substrate-free enzyme.

The Influence of Enzyme Concentration

The inactivation dose D_{37}, i.e. the dose that must be absorbed in order to inactivate the enzyme to 1/e of its original activity (cf. Ref.[8]), was determined for the substrate-free enzyme in the presence of $0.2\,mM\,DTT$ at various enzyme concentrations ($0.075 \leq c \leq 14.3$ mg/ml). The results are shown in Fig. 2. As can be seen, the data can be fitted very well by a straight line having a positive slope; this amounts to 52.5 krd ml mg^{-1}. This finding corroborates the indirect effect of

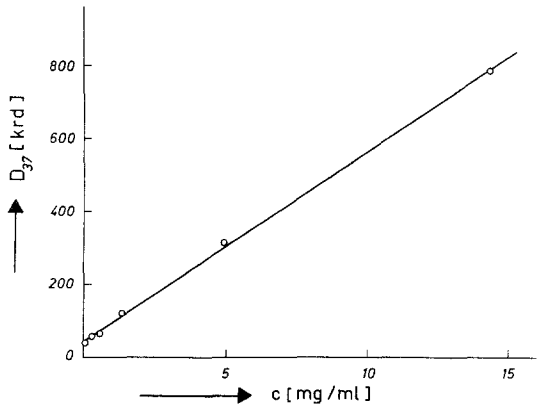

Fig. 2. The inactivation dose D_{37} of substrate-free malate synthase in the presence of $0.2\,mM\,DTT$ as a function of the enzyme concentration

radiation. The slope of the straight line corresponds to a G value (cf. Ref.[8]) for the inactivation of malate synthase of $0.099\,h\mathrm{eV}^{-1}$. It should be noted that the straight line has an intercept on the ordinate which amounts to 42 krd. Obviously this reflects the protection of the enzyme against inactivation as provided by components of the solution, probably by *DTT*. According to Fig. 2, the D_{37} for an enzyme solution with a concentration of 0.38 mg/ml as used in one of the aforementioned experiments (cf. Fig. 1b) should be about 62 krd, whereas the experiment itself had yielded only 39 krd. It seems reasonable to ascribe this discrepancy to differences in the concentration of reduced *DTT*.

The Influence of Dose Rate

In order to study the influence of the absorbed dose rate on the inactivation rate, the intensity of the X-radiation was varied by varying the tube current between 4 and 30 mA. Varying the voltage or

using filters for the attenuation of the radiation could not be applied because these procedures would have changed the spectral distribution of the radiation. To be sure that there is a linear relation between the current and the intensity of the X-ray beam, measurements of the intensity of primary radiation were performed in the *Kratky* camera by using a calibrated Lupolen platelet[9]. These experiments confirmed that

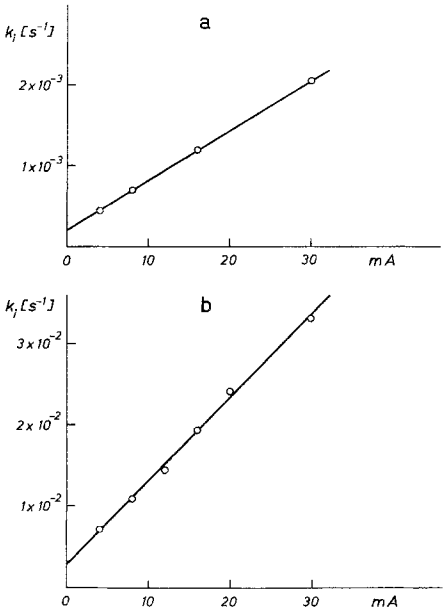

Fig. 3. The rate constants k_i of radiation-induced inactivation of substrate-free malate synthase ($c \approx 0.075$ mg/ml; 0.2 *mM DTT*) in dependence upon the electric current through the X-ray tube. *a* irradiation in the large cell, $D/t \approx 86$ rd/s at 30 mA; *b* irradiation in the small cell, $D/t \approx 1.3$ krd/s at 30 mA

the intensity of the radiation varies linearly with the current. Linear extrapolation to zero current yielded an intensity equal zero.

Fig. 3 presents the results from inactivation experiments at various tube currents. In Fig. 3a, the rate constants as obtained from measurements in the large cell are plotted versus the current, Fig. 3b shows the corresponding data as derived from measurements in the small cell. Both sets of data can be fitted very well by straight lines which do not go through the origin, however, but have positive intercepts on the ordinate.

Apparently, the rate constants of inactivation are composed of two

terms. One term is a constant, whereas the other is a function of the tube current. The constant term suggests that inactivation may occur even in the absence of radiation. Nevertheless this inactivation would have to be induced also by irradiation, since enzyme samples that had not been exposed to X-rays showed no decrease of activity during a time much longer than that covered by our experiments. This inactivation cannot be caused by any manipulations other than irradiation performed with the enzyme solutions in the course of the experiment. This was convincingly shown when an enzyme sample of the same concentration was treated in the same way as in the aforementioned experiment, except that the window of the X-ray tube was not opened. This enzyme suffered no inactivation during a stay in the small cell for 30 minutes, whereas the limiting value for the rate constant at zero current according to Fig. 3b would imply an almost complete inactivation during the same time. Furthermore, it was observed that irradiated enzyme suffered a considerable decrease of activity even some time after the stop of irradiation.

2. Inactivation and Aggregation

Irradiation Experiments

Inactivation and aggregation can only be correlated if the enzyme activity is determined before and after the aggregation experiment. Enzymic tests after the SAXS measurements cannot be conclusive, because only a small portion of the enzyme solution filled into the *Mark* capillarly is actually irradiated (cf. Ref.[2]) and gives rise to the scattering phenomenon. It is impossible to use only this part of the solution for the enzymic test. We could only extract the entire solution quantitatively from the capillary and determine the activity therefrom. Even if the volume fraction of the irradiated portion of the solution is known, we could only estimate the activity of this portion under the assumption that the activity of the not directly irradiated portion did not change during the SAXS measurement.

Therefore we chose another way to solve the problem. The solution of the enzyme or of enzyme-substrate complexes was irradiated in the small cell. The entire volume of the solution was X-irradiated with a defined dose rate for a definite time. Afterwards an aliquot of the irradiated solution was used for the enzymic test, while the main portion was diluted to a final concentration of about 7 mg/ml and was used for the SAXS experiment. Due to a pulse rate as low as 40,000 pulses counted for each point, the measurement of the innermost part of the scattering curve, which contains most of the information about the extent of aggregation, took less than one hour. This is a reasonably

Table 1. *Residual activities A_t/A_0, inactivation doses D_{37} and mean molecular parameters (mean radius of gyration $\bar{R}_p$, mean molar mass $\bar{M}_p$, and mean degree of aggregation $\bar{x}_p$) of substrate-free malate synthase and of various enzyme-substrate complexes after X-irradiation in the small cell with a dose rate $D/t = 48$ krd/min*

Sample	c (mg/ml)[a]	t (min)	A_t/A_0	D_{37} (krd)	$\bar{R}_p$ (nm)[b]	$\bar{M}_p$ (g/mol)[b]	$\bar{x}_p$ [b, c]
substrate-free enzyme	14.3	25	0.222	790	13.4	599,000	3.21
[enzyme · acetyl-CoA]	13.65	40	0.086	780	11.9	363,000	1.94
[enzyme · glyoxylate]	13.65	40	0.568	3400	9.8	246,000	1.32
[enzyme · pyruvate]	13.65	40	0.256	1400	5.4	178,000	0.95

[a] These concentrations were used for the irradiation experiments; for the scattering experiments only half the concentrations were used.

[b] Determined from the distance distribution function $p(r)$.

[c] Calculated from $\bar{M}_p$ and the molar mass of the native substrate-free enzyme[10].

short time to guarantee the scattering curve to represent a snapshot of the state of aggregation at the beginning of the SAXS measurement. Some results of these experiments are summarized in Table 1.

The Pre-Irradiated Substrate-Free Enzyme

The residual activity of 22.2% as found for the substrate-free enzyme after irradiation corresponds to a D_{37} of 790 krd. This result was

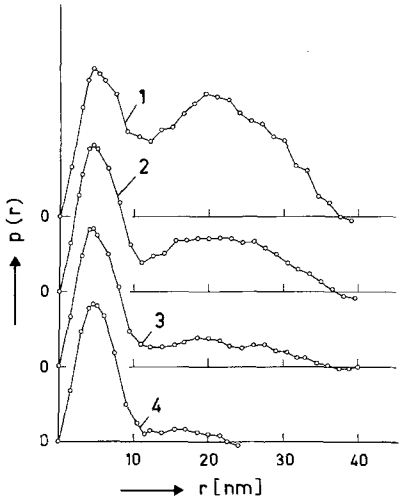

Fig. 4. Distance distribution functions $p\ (r)$ of substrate-free malate synthase and of various enzyme-substrate complexes after X-irradiation of the solutions ($c \approx 14$ mg/ml; 0.2 *mM DTT*) in the small cell with a dose rate of 48 krd/min. *1* substrate-free enzyme, time of irradiation $t = 25$ min; *2* [enzyme · acetyl-CoA], $t = 40$ min; *3* [enzyme · glyoxylate], $t = 40$ min; *4* [enzyme · pyruvate], $t = 40$ min. Enzyme concentrations for the SAXS-measurements amounted to about 7 mg/ml

already shown in Fig. 2. The mean radius of gyration $\bar{R}_G$ of 12.2 nm and the mean molar mass $\bar{M}_G$ of 568,000 g/mol, as derived from the *Guinier* plot, are much larger than the data for the native unirradiated substrate-free enzyme, namely $R = 3.95_5$ nm and $M = 186{,}800$ g/mol (cf. Ref.[10]). However, the values for $\bar{R}_G$ and $\bar{M}_G$ must be considered to be still underestimated since no extrapolation of the scattering curve to zero concentration could be performed.

The distance distribution function $p\,(r)$ (Fig. 4, curve 1) shows the presence of a considerable amount of aggregates in the solution. The $p\,(r)$ function is positive up to distances as large as about 37 nm. This value represents the maximum visible diameter of the aggregates. The

shape of the $p(r)$ function is different from those obtained from our previous scattering experiments (Part I), where the aggregation occurred in the capillary under the conditions of the SAXS experiment. The $p(r)$ function has two distinct maxima which are separated by a relatively deep minimum. The higher maximum at the smaller distances may be due to unaggregated enzyme and due to contributions from smaller aggregates. The broad maximum at the large distances must be caused by higher amounts of larger aggregates as compared to the aggregates in our previous studies. This follows from the fact that the previously obtained $p(r)$ functions contained only one distinct maximum namely at smaller distances. This maximum broadened with proceeding aggregation towards larger distances, and at the most advanced stages of aggregation a shoulder appeared at about the same distances where now the minimum occurs.

One possible explanation for the difference between the $p(r)$ functions shown in Fig. 4 and in Part I may be the difference in the dose rates (about 50 krd/min for the irradiation in the small cell, about 1 krd/min for irradiation during SAXS). Moreover, in the small cell the entire solution was irradiated, whereas in the previous SAXS experiments only a thin zone of the sample was irradiated. Thus the energy absorbed by this zone was certainly dissipated over the much larger volume of the capillary by means of convection and/or diffusion of radiolysis products. Furthermore the time needed for the SAXS experiments was about 100 times longer than that for the irradiation experiments in the small cell. Under these conditions also the diffusion of unirradiated enzyme into the irradiation zone of the capillary and of irradiated enzyme out of the irradiation zone during the SAXS experiment was possible. This may explain the occurrence of smaller aggregates in our previous SAXS experiments.

The determination of the mean radius of gyration and mean molar mass of the irradiated substrate-free enzyme by using the $p(r)$ function led to values of $\bar{R}_p = 13.4$ nm and $\bar{M}_p = 599{,}000$ g/mol, which are both larger than the values determined from the *Guinier* plot. We give preference to the values derived from the $p(r)$ function and include only these values in Table 1, because they are certainly influenced by the neglection of interparticular interferences to a much lesser extent than the data obtained from the *Guinier* plot. The quotient of $\bar{M}_p$ of the irradiated enzyme divided by M of the native enzyme yields $\bar{x}_p = 3.21$.

The cross-section *Guinier* plot of the scattering curve of the pre-irradiated substrate-free enzyme (Fig. 5, curve 1) also differs from the cross-section *Guinier* plots obtained previously for the highly aggregated enzyme (Part I). There is only a narrow angular range where the curve can be fitted by a straight line. The slope of the line corresponds

to $\bar{R}_c = 2.72\,\text{nm}$. This value is almost identical to $R_c = 2.73\,\text{nm}$ as obtained previously for the unaggregated enzyme. In the innermost portion the intensity decreases first considerably and only increases at the smallest angles. Obviously this shape of the curve does not reflect the presence of rodlike particles.

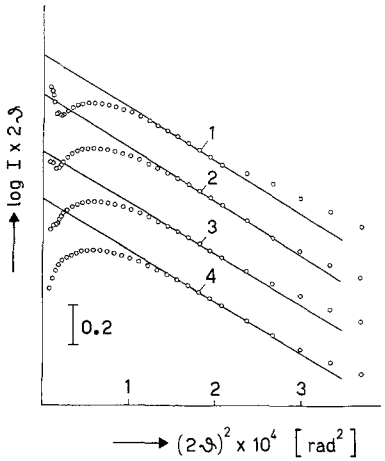

Fig. 5. Cross-section *Guinier* plots of scattering curves of pre-irradiated substrate-free malate synthase and of pre-irradiated enzyme-substrate complexes (cf. legend to Fig. 4). The following mean cross-sectional radii of gyration can be derived from the curves: *1* substrate-free enzyme, $\bar{R}_c = 2.72\,\text{nm}$; *2* [enzyme · acetyl-CoA], $\bar{R}_c = 2.73\,\text{nm}$; *3* [enzyme · glyoxylate], $\bar{R}_c = 2.66\,\text{nm}$, *4* [enzyme · pyruvate], $\bar{R}_c = 2.67\,\text{nm}$

The Pre-Irradiated Enzyme-Substrate Complexes

The irradiated [enzyme · acetyl-CoA] complex was found to have a residual activity of only 8.6%. Taking into account the longer time of irradiation (cf. Table 1), this value corresponds to almost the same D_{37} as for the substrate-free enzyme, namely 780 krd. This result corroborates the aforementioned finding that acetyl-CoA does not inhibit the inactivation of the enzyme. Nevertheless, acetyl-CoA inhibits the aggregation of the enzyme as follows from the lower values for $\bar{R}_p$, $\bar{M}_p$ and $\bar{x}_p$ as compared to the pre-irradiated substrate-free enzyme and from the $p(r)$ function shown in Fig. 4 (curve 2). The maximum of this function at larger distances is much lower than in the curve for the pre-irradiated substrate-free enzyme (curve 1), in spite of the much longer time of irradiation. However, the first zero is at about

the same distance as the first zero in the $p(r)$ function of the pre-irradiated substrate-free enzyme. The cross-section *Guinier* plot of the scattering curve of the [enzyme · acetyl-CoA] complex (Fig. 5, curve 2) has great similarity with the corresponding curve for the pre-irradiated substrate-free enzyme, except that the increase of intensity at the smallest angles is not so pronounced.

The residual activity of the irradiated [enzyme · glyoxylate] complex of 56.8% corresponds to a D_{37} as high as 3.4 Mrd. Both the values for the molecular parameters $\bar{R}_p$, $\bar{M}_p$ and $\bar{x}_p$ of the [enzyme · glyoxylate] complex in Table 1 and the shape of the $p(r)$ function in Fig. 4 (curve 3) suggest that the amount of aggregates is lower than in the case of the substrate-free enzyme or of the [enzyme · acetyl-CoA] complex. The first zero in the $p(r)$ function, however, is still at a distance comparable to that for the pre-irradiated substrate-free enzyme. In the cross-section *Guinier* plot of the scattering curve of the [enzyme · glyoxylate] complex (Fig. 5, curve 3), there is no increase of intensity towards the smallest angles, only a kind of shoulder is indicated at about 3.5 mrad.

The residual activity of the irradiated [enzyme · pyruvate] complex was found to be lower than that of the irradiated [enzyme · glyoxylate] complex, namely 25.6%, and the D_{37} is 1.4 Mrd. Nevertheless, the $p(r)$ function (Fig. 4, curve 4) shows that the extent of aggregation must be very low. The $p(r)$ function goes through zero already at about 23 nm. The value of $\bar{R}_p = 5.4$ nm is still larger than R of the native enzyme, however, $\bar{M}_p$ is found to be even slightly smaller than M of the native substrate-free enzyme particle. Accordingly, $\bar{x}_p$ is slightly smaller than unity. This behaviour could be due to the neglection of interparticulate interferences, however, it could reflect a degradation of enzyme particles as well. The cross-section *Guinier* plot of the scattering curve of the pre-irradiated [enzyme · pyruvate] complex (Fig. 5, curve 4) strongly resembles the curves obtained for the early steps of aggregation in our previous experiments with substrate-free enzyme (cf. Part I).

Remarks on the Thickness *Guinier* Plots

The thickness *Guinier* plots of the scattering curves of the pre-irradiated substrate-free enzyme and the pre-irradiated enzyme-substrate complexes (Fig. 6) show the occurrence of a pseudo thickness factor. Up to an angle of about 22 mrad the curves are very similar to each other. At larger angles, however, particularly in the region beyond 30 mrad, the curves for the enzyme-substrate complexes (curves 2-4) differ considerably from the curve for the substrate-free enzyme

(curve 1); they show a distinct minimum at about 35 mrad which does not appear in curve 1. The approximation of the curves by straight lines is somewhat ambiguous and therefore the $\bar{R}_t$ values are certainly of low accuracy; they range from $\bar{R}_t = 0.91$ nm for the [enzyme · pyruvate] complex to $\bar{R}_t = 1.14$ nm for the [enzyme · acetyl-CoA] complex.

The oscillations of the curves around the straight lines are more pronounced than the oscillations found in our previous studies on

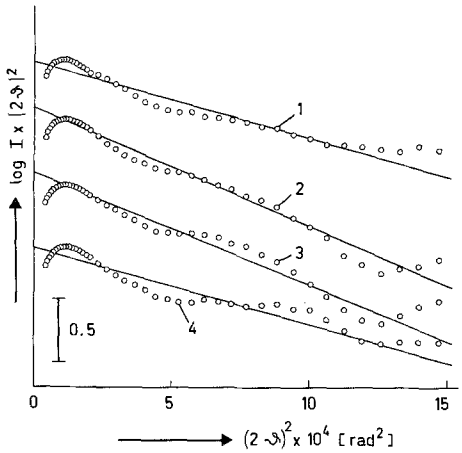

Fig. 6. Thickness *Guinier* plots of scattering curves of pre-irradiated substrate-free malate synthase and of pre-irradiated enzyme-substrate complexes (cf. legend to Fig. 4). The following mean radii of gyration of the thickness can be derived from the curves: *1* substrate-free enzyme, $\bar{R}_t = 0.92$ nm; *2* [enzyme · acetyl-CoA], $\bar{R}_t = 1.14$ nm; *3* [enzyme · glyoxylate], $\bar{R}_t = 1.10$ nm; *4* [enzyme · pyruvate], $\bar{R}_t = 0.91$ nm

aggregating substrate-free enzyme which were performed at enzyme concentrations about 4 times as large as in the present study. It cannot be ruled out that part of the discrepancies between the tail ends of the curves are caused by experimental errors due to the relatively low enzyme concentrations used in the present study. The meaning of the minimum at about 35 mrad in the curves 2-4 is not quite clear. The theoretical scattering curves for the native enzyme particle show an inflection but not a minimum in this angular range. Also in our previous measurements on aggregating substrate-free enzyme no minimum could be observed. One possible explanation for the missing of such a minimum in experimental curves would be the presence of small

degradation products which would lead to a flattening of the tail ends of the scattering curves in the thickness *Guinier* plots (cf. Ref.[3]). The occurrence of such fragmentation products on X-irradiation was confirmed by electrophoretic studies[11,4].

Discussion and Conclusions

As was convincingly demonstrated by our experiments, the enzyme malate synthase suffers radiation damages upon X-irradiation in aqueous solution. These damages include the formation of aggregates and possibly of fragments, as well as the loss of enzymic activity.

The inactivation of an enzyme by X-irradiation in aqueous solution may have several reasons. For instance, the enzyme can lose its activity by damages that occur at the active centre itself or by damages at other parts of the enzyme molecule which influence the active centre by structural changes. These structural changes would also include the formation of covalent or non-covalent cross-links or the degradation of parts of the enzyme. The linear dependence of the inactivation dose D_{37} on the enzyme concentration (cf. Fig. 2) suggests that the indirect effects of radiation, i.e. the reaction of radical and non-radical products of water radiolysis with the enzyme, are mainly responsible for radiation damages.

Because of the presence of oxygen in our enzyme solutions the radicals $H^{\cdot}$ and e_{aq}^{-} will contribute only little to inactivation and aggregation of malate synthase, in contrast to $OH^{\cdot}$ and possibly to O_2^{-} and $HO_2^{\cdot}$ radicals and H_2O_2 (cf. Refs.[12–19]).

In proteins especially amino acids with sulfur or aromatic rings are subject to radiation damage (cf. Refs.[20,21]). Malate synthase was shown to contain a lot of cys, met, tyr, trp, phe[22,23]; cys and trp were found to be correlated with substrate binding and/or enzymic activity[7,23].

The sensitivity of sulfhydryl groups of enzymes to X-irradiation was established e.g. with glyceraldehyde-3-phosphate dehydrogenase or papain[24–26,17,18]. In the case of malate synthase a similar sensitivity of the sulfhydryl groups to X-irradiation may be expected. Indeed, X-irradiation of the enzyme caused a loss of sulfhydryl groups[2]. Polyacrylamide gel disc electrophoreses in the presence or absence of sodium dodecyl sulfate established the formation of covalent and possibly of non-covalent cross-links in irradiated malate synthase[11,4]. Part of the covalent cross-links were interpreted to be disulfide bridges. The electropherograms also clearly reflected the formation of various protein fragments. The observation of an inactivation (repairable by *DTT*) after addition of H_2O_2 to unirradiated enzyme and the similarity of the electropherograms of H_2O_2-treated enzyme and those of

X-irradiated enzyme suggest a possible role of H_2O_2 in the X-ray induced inactivation and aggregation of malate synthase[2,4,11].

1. Protective Effects

It was clearly shown by our inactivation experiments that the presence of *DTT* in the reduced form, of the substrate glyoxylate or of the substrate analogue pyruvate protects the enzyme against X-ray induced inactivation. Both *DTT* in the reduced and oxidized form, the substrates glyoxylate or acetyl-CoA or the substrate analogue pyruvate, as well as ethanol were shown by SAXS to protect the enzyme against X-ray induced aggregation. A protective effect of *DTT* against X-ray damage of malate synthase was also shown by electrophoretic studies[11,4].

As explanations for the protective effects may be mentioned scavenging of radicals, reactions with non-radical agents produced by irradiation, shielding of sensitive groups by specific or unspecific binding to the enzyme, conformational changes of the enzyme and its active site, repair of radiation damage etc. (cf. Ref.[2]).

The Protective Effect of Dithiothreitol

The radioprotection of proteins by *DTT* has been subject of several investigations[17,27–30]. It was found that upon irradiation in aqueous solution *DTT* may undergo a variety of reactions: *DTT* (in the reduced form) may react with OH (reaction 1), and also with O_2^- thereby releasing H_2O_2 (r. 2); the cyclic *DTT* radical formed by these reactions may react with molecular oxygen to oxidized *DTT*, releasing O_2^- (r. 3); oxidized *DTT* is also formed in a slow reaction of reduced *DTT* with H_2O_2 (r. 4); oxidized *DTT* may be reduced to the cyclic *DTT* radical by H and e_{aq}^- (r. 5). By reactions 2 and 3, a chain oxidation of *DTT* may take place. The increased formation of H_2O_2 in the presence of *DDT* (through reaction 2) was also confirmed by own experiments[4]. In the presence of an enzyme containing sulfhydryl groups (which are damaged not only by OH but also by O_2^- and H_2O_2) the situation becomes even more complicated.

Therefore the protective effect of *DTT* against radiation damage may be caused by OH scavenging and various repair mechanisms including the repair of sulfenic acid products formed by H_2O_2[17–19,30]. *Lin* et al.[17] have found that in the case of papain the amount of non-repairable inactivation is increased by *DTT*, while the amount of total inactivation is considerably decreased.

The protective effect of *DTT* against aggregation of malate synthase is provided by *DTT* in the reduced form and, to a certain extent, also by oxidized *DTT*, in contrast to inactivation where oxidized *DTT* showed no protective effect. The further investigation of this different behaviour and a differentiation between repairable and non-repairable inactivation will be a subject of further experiments.

The Protective Effects of Substrates

The thioester acetyl-CoA and the α-ketoacid anions glyoxylate and pyruvate might also be attacked by the radical products of water radiolysis or by H_2O_2. The reaction of H_2O_2 with pyruvate yields acetate, the reaction with glyoxylate leads to formate. Formate is a scavenger of $OH\cdot$, in the presence of oxygen O_2^- is released (cf. Ref.[31]). We found in irradiated aqueous solutions of the substrates or of the analogue a reduced concentration of H_2O_2 upon X-irradiation, as compared to irradiated water. Irradiation for 50 min in the large cell yielded the following H_2O_2 contents: $4 \times 10^{-4}\%$ for water, $0.8 \times 10^{-4}\%$ for 0.1 *M* glyoxylate, $0.5 \times 10^{-4}\%$ for 0.1 *M* pyruvate, $2.8 \times 10^{-4}\%$ for 6.2 *mM* acetyl-CoA.

Our SAXS experiments on pre-irradiated enzyme-substrate complexes established the following series of efficiency for protection against aggregation: pyruvate > glyoxylate > acetyl-CoA. The same series was obtained for the efficiency of the substrates for the inhibition of H_2O_2 formation. On the other side, a somewhat different series resulted for the protection against inactivation, namely glyoxylate > pyruvate ≫ acetyl-CoA; here acetyl-CoA did not show any protective effect.

It is unlikely that the inefficiency of acetyl-CoA to inhibit the inactivation of the enzyme is only due to the lower molar concentrations of this substrate (2 or 3 *mM*), as compared to glyoxylate or pyruvate (5 or 50 *mM*), because at the same low concentrations acetyl-CoA did provide protection of the enzyme against aggregation.

Possibly the assumption of a shielding of the binding sites of the enzyme by the substrates and a different sensitivity of the binding sites to X-irradiation is able to explain the remarkable difference of the protective effects provided by the two groups of substrates (i.e. glyoxylate or pyruvate on one side, acetyl-CoA on the other side). In this case the series of efficiency for the protection against inactivation suggests that the binding site for acetyl-CoA is less sensitive to X-irradiation than that for glyoxylate or pyruvate. In this context it may be mentioned that an involvement of trp in the binding of glyoxylate or pyruvate, but not in the binding of acetyl-CoA, was demonstrated by previous CD-spectroscopic and fluorescence studies on unirradiated malate synthase[7].

A specific protection of the enzyme by the bound substrates would depend on the degree of saturation. Therefore different changes of saturation, as caused by different damages of the substrates by reaction with the products of water radiolysis, might serve as another explanation for the different efficiency against inactivation.

Lactate dehydrogenase has also been demonstrated to be

specifically protected against radiation induced inactivation by pyruvate or lactate[32] or by NAD^+[14].

The Protective Effect of Ethanol

Similar protective effects of ethanol as found for malate synthase have been reported for the X-ray induced inactivation of ribonuclease[33,34]. Scavenging of $OH^{\cdot}$ radicals or reaction of ethanol radicals with the enzyme might serve as an explanation for this protective effect.

2. Correlation of Inactivation and Aggregation

According to our two-dimensional aggregation model, which has been deduced from the SAXS studies, the cross-linking between damaged enzyme particles would have to take place preferably at definite sites. These sites must be definite amino acid residues[35] located at the periphery of the oblate enzyme particles. Since the substrate binding sites of malate synthase are also assumed to be situated near the periphery of the molecule[36] an interdependence between aggregation and inactivation may be expected.

Nevertheless our results for pre-irradiated samples of substrate-free enzyme and enzyme-substrate complexes convincingly demonstrate that the extent of inactivation does not necessarily depend on the extent of aggregation. This conclusion can be drawn from the fact that both the pre-irradiated substrate-free enzyme and the pre-irradiated [enzyme · pyruvate] complex have a residual activity of about one quarter of the activity of unirradiated substrate-free enzyme, though they differ so much in the extent of aggregation. The pre-irradiated substrate-free enzyme has a mean degree of aggregation of about 3 (cf. Table 1) while that of the pre-irradiated [enzyme · pyruvate] complex amounts to about 1. On the other hand, the residual activity of the pre-irradiated [enzyme · acetyl-CoA] complex, which has a mean degree of aggregation of about 2, is less than 10%. Therefore it can be assumed that the binding sites for cross-linking are not necessarily identical with the binding sites for the substrates or with the essential sulfhydryl groups.

3. Implications for Conventional Small-Angle X-Ray Scattering Investigations

The possible influence of X-irradiation on the structure of biopolymers during the performance of a SAXS experiment has been ignored usually. Only a few authors mentioned a possible influence of radiation damage and/or denaturation.

SAXS experiments on hemocyanin from *helix pomatia*[37] caused a

change of the colour of the sample in the zone hit by the X-ray beam; however this change was not accompanied by an alteration of the scattering curves during the time covered by the SAXS experiment. SAXS on yeast glyceraldehyde-3-phosphate dehydrogenase as a function of saturation with NAD^+ at 40 °C[38,39] established a considerable change of scattering curves, particularly in the case of the apoenzyme, as a function of measuring time, a phenomenon which was interpreted to reflect an aggregation process. This process was accompanied by a decrease of enzymic activity, and led finally to a visible turbidity of the enzyme solution. At that time it was assumed that this behaviour is primarily due to heat denaturation. In the light of the above considerations it seems plausible that there may occur additionally an X-ray induced damage upon prolonged exposure of the enzyme to X-rays (cf. Ref.[40]).

The first detailed investigation of an X-ray induced alteration of a biopolymer during a SAXS measurement has been performed in the case of the enzyme malate synthase, as has been described in preliminary reports by us[2,41–47] and in a detailed way in this paper and in Part I.

Two general aspects of our results on the radiation effects of malate synthase have to be discussed. The first aspect is concerned with the occurrence of radiation damages which can affect considerably the SAXS investigations of biopolymers. This, of course, will be the most frequent confrontation with X-ray damage in the practice of SAXS. The second aspect is, that SAXS can be used successfully for the structural characterization of biopolymers which have been damaged by X-rays on purpose. This approach is of particular interest in the field of radiation biology.

For the detailed structural characterization of biopolymers, especially for the investigation of small structural changes (e.g. upon ligand binding, upon changes of *pH* or temperature, etc.) it is necessary to suppress or at least to reduce the noxious influence of radiation damage. This can be achieved by the choice of appropriate conditions for the SAXS experiments. As was shown by our experiments and investigations of others, a stabilization of biopolymers against X-ray damage can be provided under certain conditions by addition of small amounts of various substances to the solution under investigation, namely sulfur containing compounds (mercaptanes, cysteamine, thiourea, *DTT*, mercaptoethanol, cysteine, cystine, glutathione), *EDTA*, alcohols (e.g. ethanol, *t*-butanol), sodium formate, substrates and substrate analogues, coenzymes etc. (cf. e.g.[14,17,28,30,32,33,48–51]). High salt concentrations were also reported to suppress aggregate formation[52]. Sometimes the presence of oxygen in solution has been

observed to impede aggregation[53,54], whereas in other cases the presence of oxygen was reported to lead to an enhancement of radiosensitivity; thus a replacement of oxygen by other gases (e.g. nitrogen or argon) may supply a protective effect (e.g.[18]).

Besides a protection of the biomolecule on the chemical level, some apparative or procedural precautions can be applied too, in order to reduce radiation damage. It is self-evident that samples should not be exposed to higher X-ray doses than needed for the performance of the SAXS experiment. Unfortunately this condition is not always fulfilled; for instance in many SAXS experiments the samples are irradiated with the entire spectrum of the X-ray tube (white radiation plus characteristic lines), though only the scattering of a single line (e. g. CuKα line) is evaluated and interpreted. The use of a monochromator would drastically suppress radiation damage. Another possibility to reduce considerably the exposure of the sample to X-irradiation would be the application of a shutter which prevents the primary beam from hitting the sample during all, usually slow, motions of the goniometer (not only during the back-motion as in the present version of the step scanning device) so that the sample is irradiated only for the time needed to measure the scattered radiation. A possible influence of the dose rate should also be taken into account in future work. Very sensitive biomolecules may be successfully investigated by use of a special flow device[39] or by a frequent exchange of the irradiated sample by an unirradiated sample.

From the viewpoint of SAXS, radiation damages which do not alter the scattering curves can be considered to be harmless. A noxious influence of radiation damage on the scattering curves can be ruled out by repeated scanning, if the curves are found to be quantitatively unchanged. Therefore radiosensitive samples should preferably be investigated by repeated scanning of scattering curves in short runs with low counting rates, instead of one measurement extending over several hours. A consequent application of this precaution requires also a sophisticated evaluation procedure[10,44].

Due to the weak absorption of neutrons by matter, radiation damage does not play an important role in small-angle neutron scattering. But the limited availability of suitable neutron sources and the lower accuracy in the outer part of small-angle neutron scattering curves, as compared to SAXS curves, does not allow replacement of SAXS by small-angle neutron scattering in all cases.

4. Application of Small-Angle X-Ray Scattering in Radiation Biology

Radiation biology and the structural analysis by means of SAXS are fields of research which have developed separately in the past. The

use of the SAXS technique in the field of radiation biology offers a variety of novel applications[2]. These applications include the simultaneous production of damaged particles by the impact of the primary radiation and structural characterization by measurement of the scattered radiation, or the investigation and characterization of radiation products obtained by irradiation of biopolymers prior to the SAXS experiment. While the first approach delivers information on structural changes of the irradiated samples and on the kinetics of processes induced by X-irradiation, the second approach, particularly when it is combined with separation techniques or biological tests, may yield detailed information on the structure and function of damaged particles. In so far, the application of SAXS to the field of radiation biology can offer further insight into the biochemistry of biopolymers.

The monitoring of X-ray induced aggregation by means of SAXS has also been applied meanwhile by us to other proteins (glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, ribonuclease, serum albumin); the results from these studies were compared with those from malate synthase[40].

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