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RADIATION EFFECTS ON AN ALLOSTERIC ENZYME WITH TWO CATALYTIC ACTIVITIES

X-RAY INACTIVATION OF THE THREONINE-SENSITIVE ASPARTOKINASE-HOMOSERINE DEHYDROGENASE FROM *ESCHERICHIA COLI*

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SUMMARY

The threonine-sensitive aspartokinase–homoserine dehydrogenase (ATP: L-aspartate 4-phosphotransferase, EC 2.7.2.4; L-homoserine: NAD oxidoreductase, EC 1.1.1.3) from *Escherichia coli* was irradiated in dilute aqueous solution in the presence of air with X-rays at 25°.

The two enzyme activities were destroyed as exponential functions of the radiation dose, the *G* values being 0.006 and 0.015 for the dehydrogenase and the kinase activities, respectively. The sensitivity of the dehydrogenase activity to inhibition by threonine was inactivated with a *G* value of 0.015, whereas the corresponding sensitivity of the kinase activity was unaffected by irradiation.

The enzyme sulfhydryl groups were destroyed with a *G* value of 0.25.

The radiation-induced loss of the sensitivity of the dehydrogenase activity to threonine was effectively protected by the presence of threonine during irradiation, whereas the dehydrogenase activity as such was protected only to a limited extent.

Kinetic studies indicated that the radiation-induced loss of the allosteric dehydrogenase function is an all-or-none process.

The results indicate that destruction of sulfhydryl groups plays an important role in the radiation-induced loss of the allosteric dehydrogenase function and the catalytic kinase function. The mechanisms probably involve dissociation of the enzyme.

INTRODUCTION

It has recently been found that certain enzymes are capable of catalyzing two

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Abbreviation: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid).

or more different reactions[1–5]. An interesting example is the threonine-sensitive aspartokinase–homoserine dehydrogenase (ATP: L-aspartate 4-phosphotransferase, EC 2.7.2.4; L-homoserine: NAD oxidoreductase, EC 1.1.1.3) from *E. coli*. This enzyme catalyzes the first and the third steps in the conversion of aspartate to threonine[5].

$$\text{Asparate} \xrightarrow{(1)} \text{aspartylphosphate} \xrightarrow{(2)} \text{aspartate semialdehyde} \xrightarrow{(3)} \text{homoserine} \xrightarrow{(4)} \text{homoserine phosphate} \xrightarrow{(5)} \text{threonine}$$

The structure and kinetic properties of the enzyme have been extensively studied[5–11]. The enzyme complex consists of six subunits which seem to have equal molecular weights[10]. The aspartokinase activity, as well as the allosteric inhibition of the dehydrogenase activity by threonine, depend on sulfhydryl groups[7,8].

In the present paper the inactivation of the enzyme by X-rays has been investigated. The purpose was to study the extent and the mechanism of the radiation inactivation of the different functions of this complex enzyme. Studies of the X-ray inactivation of an allosteric enzyme having two different catalytic functions have not been previously reported.

MATERIALS AND METHODS

Materials

ATP and NADPH were purchased from Boehringer & Soehne, Mannheim, Germany. DL-Allylglycine, L-aspartate, DL-threonine and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co., St. Louis, Mo. Sephadex G-25 and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala, Sweden. All chemicals used were of the highest purity.

Aspartate semialdehyde was prepared from DL-allylglycine by ozonolysis according to the method of BLACK AND WRIGHT[12]. The aspartate semialdehyde solution was kept at −10° in 4 M HCl. Immediately before use it was neutralized with KOH.

Preparation of enzyme

The enzyme was prepared from *E. coli* K 12, Strain HfrH. The bacteria were grown at 37° under vigorous aeration on minimal medium containing 10 g of glucose and 0.5 mg thiamine per l (refs. 5,8). The culture was harvested after approx. 3/4 of the log period, and the enzyme was prepared as described by TRUFFA-BACHI *et al.*[8], with the following modification. The homogenization was carried out in the presence of 150 g glass beads per 50 g of wet bacteria with the use of an Omnimixer. In the last step the enzyme was passed twice through the DEAE-Sephadex A-50 column, instead of a hydroxyapatite column. The enzyme was kept in suspension at room temperature in a 50% saturated $(NH_4)_2SO_4$ solution. The specific activity of the dehydrogenase function of the enzyme was 27 units/mg (ref. 12), and an inhibition of 70–75% was obtained with 10 mM threonine. The allosteric inhibition decreased upon storage of the enzyme.

The purity of the enzyme was checked by disk electrophoresis in 7.5% polyacrylamide gel[13] containing 0.3% DL-threonine. Only one main band was found.

Assay of enzyme activity

The dehydrogenase activity was measured by the method of PATTE *et al.*[6] using aspartate semialdehyde as substrate. The assay mixture contained (total volume 1 ml): 1 mM DL-aspartate semialdehyde, 0.8 M KCl, 40 mM Tris–HCl (pH 7.5) and 0.3 mM NADPH. The reaction was started by adding the enzyme. The reaction rate was measured with a Unicam SP 800 at 340 nm.

The kinase activity was measured as described by STADTMAN *et al.*[14], except that mercaptoethanol was omitted. The assay mixture contained (total volume 1.0 ml): 10.4 mM ATP, 94 mM Tris–HCl (pH 8.1), 16 mM $MgSO_4$, 10 mM L-aspartate, 0.8 M NH_2OH, and 0.8 M KCl. The reaction was started by the addition of the enzyme. After incubation at 24° for 30 min the reaction was stopped by the addition of 1.0 ml of $FeCl_3$ reagent[15]. After centrifugation, the absorbance of the aspartate–hydroxamate–iron complex was measured at 540 nm in a Zeiss spectrophotometer.

The protein was determined by the method of LOWRY *et al.*[16]. A molecular weight of 360 000 was used[8,10].

Irradiation conditions

To 50 μl of the enzyme suspension was added 1 ml 50% saturated $(NH_4)_2SO_4$. The suspension was centrifuged, and the precipitate dissolved in 0.2 M potassium phosphate buffer (pH 7.2). This solution was stable for at least 1 h. The enzyme solution was irradiated in glass vials in the presence of air at 25° with a Stabilipan X-ray machine. The irradiation parameters were 220 kV, 20 mA and 0.5-mm Cu filter. The dosimetry was carried out with a Fricke dosimeter.

RESULTS

Radiation sensitivity

When the enzyme was irradiated in dilute solution, the dehydrogenase activity

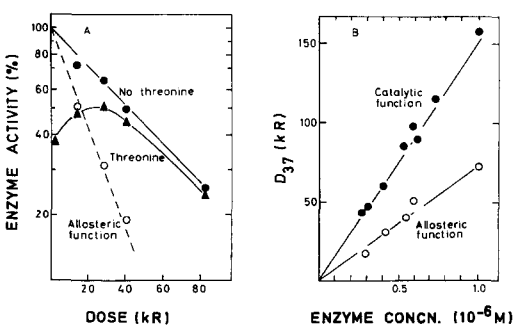

Fig. 1. X-ray inactivation of the dehydrogenase activity of the threonine sensitive aspartokinase–homoserine dehydrogenase. A. Dose–response curves in the presence and absence of 10 mM threonine during the assay. The enzyme ($0.38 \cdot 10^{-6}$ M) was irradiated at 25° in 0.2 M potassium phosphate buffer (pH 7.2) in equilibrium with air. The activity is expressed in per cent of that of the unirradiated control, measured in the absence of threonine. The inhibition by 10 mM threonine, expressed in per cent of the inhibition of the unirradiated enzyme, is taken as a measure of the remaining allosteric activity (the dashed line). B. The D_{37} dose for the catalytic and allosteric activities as functions of the enzyme concentration. The D_{37} doses were obtained from complete dose–inactivation curves.

disappeared as an exponential function of the dose (Fig. 1A). However, when the activity was measured in the presence of 10 mM threonine, an apparent stimulation of the activity was observed after small doses of radiation. After this initial increase, the activity disappeared as an exponential function of the dose, and the final slope of the curve was the same as that obtained in the absence of threonine. With increasing dose of radiation the inhibition obtained with threonine decreased. If the allosteric function is defined as the inhibition obtained by 10 mM threonine, and is set equal to 100% in the unirradiated enzyme, it is possible to calculate the effect of radiation on the allosteric function from measurements of the dehydrogenase activity in the absence and presence of threonine (the dashed line). The sensitivity to threonine was more than twice as susceptible to radiation as was the catalytic dehydrogenase activity. The relationship varied slightly in different preparations.

The yields for the inactivation of the catalytic and allosteric dehydrogenase functions were calculated from the slopes of the lines in Fig. 1B. Here, the D_{37} doses derived from the exponential dose–response curves have been plotted against enzyme concentration. The G values (the number of enzyme molecules inactivated per 100 eV absorbed by the solution) were 0.006 for the catalytic dehydrogenase activity and 0.015 for the allosteric dehydrogenase function.

The inactivation of the kinase activity of the enzyme is shown in Fig. 2A.

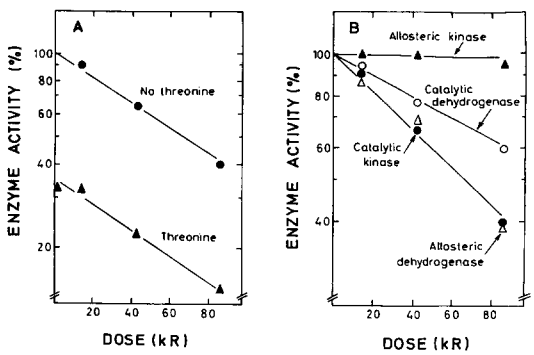

Fig. 2. A. X-ray inactivation of the kinase activity of the threonine-sensitive aspartokinase–homoserine dehydrogenase. The enzyme ($1.25 \cdot 10^{-6}$ M) was irradiated in 0.2 M potassium phosphate buffer (pH 7.2) in equilibrium with air at 25°. The activities are expressed in per cent of that of the unirradiated control, measured in the absence of threonine. B. Comparison of the X-ray sensitivity of the two catalytic and allosteric functions of the enzyme. Conditions as in A. The allosteric function was calculated as the degree of inhibition by threonine, expressed in per cent of the inhibition of the unirradiated enzyme.

Like the dehydrogenase activity, the kinase activity disappeared as an exponential function of the dose. Since high concentrations of threonine will inhibit the kinase activity completely, its sensitivity to the effector was measured at a threonine concentration of 0.7 mM, which decreased the activity of the unirradiated enzyme by approx. 70%. The slope of the inactivation curve was the same in the presence and absence of threonine, demonstrating that the X-irradiation did not affect the sensitivity of the kinase activity to threonine.

In Fig. 2B the effect of radiation on the different enzyme functions are sum-

marized. The kinase activity disappeared at the same rate as the sensitivity of the dehydrogenase to threonine.

X-ray destruction of sulfhydryl groups

In order to elucidate the mechanism of the radiation-induced inactivation of the enzyme, the destruction of sulfhydryl groups was correlated with the loss of the dehydrogenase function and its sensitivity to threonine (Fig. 3A). The X-ray de-

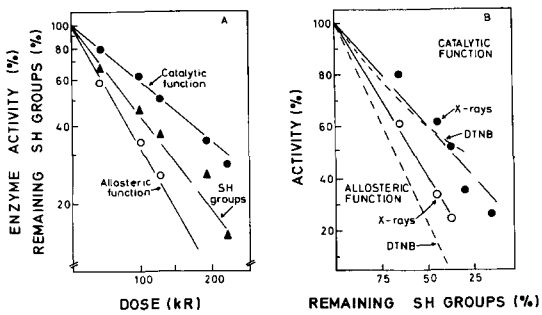

Fig. 3. Disappearance of enzyme sulfhydryl groups upon irradiation. The enzyme ($1.50 \cdot 10^{-6}$ M) was irradiated as described in the legend to Fig. 1. The dehydrogenase activity was determined in the presence and absence of 10 mM threonine, and the remaining sulfhydryl groups were titrated with DTNB[22] in the presence of 4 M urea. A. Rate of disappearance of sulfhydryl groups and the catalytic and allosteric dehydrogenase activity. B. The catalytic and allosteric dehydrogenase activity expressed as function of remaining sulfhydryl groups after X-irradiation. The X-ray data are taken from A, the DTNB data from ref. 8.

struction of sulfhydryl groups, measured in the presence of urea, follows a straight line in a semilogarithmic plot. Studies by previous authors have shown that in the presence of a denaturing agent a total of 28–30 sulfhydryl groups can be titrated, whereas in the absence of denaturing agents, only 16–18 sulfhydryl groups react with DTNB[8]. The fact that the X-ray destruction of sulfhydryl groups follows a straight line indicates that the water radicals fail to destroy selectively those sulfhydryl groups that possess the highest chemical reactivity. From the slope of the curve it can be calculated that the sulfhydryl groups were destroyed with a G value of 0.25.

From the data the dehydrogenase activity and its sensitivity to threonine have been plotted *versus* the percentage of intact sulfhydryl groups remaining (Fig. 3B). For comparison, previous results[8] obtained by blocking of sulfhydryl groups with DTNB have been included. The catalytic activity disappeared at approximately the same rate whether the sulfhydryl groups were destroyed by X-rays or blocked by DTNB. On the other hand, sulfhydryl destruction by X-rays was considerably less effective than sulfhydryl blocking by DTNB in abolishing the allosteric function. Thus, the sensitivity to threonine is completely lost when 60% of the sulfhydryl groups have been blocked by DTNB[8], while approx. 80% of the sulfhydryl groups must be destroyed by X-rays before the sensitivity is lost. These results indicate that the X-ray destruction of sulfhydryl groups is sufficiently extensive to account for the radiation-induced loss of the allosteric function.

Irradiation of an enzyme may result in numerous chemical changes. Hence,

the demonstration that the inactivation of an enzyme function is associated with destruction of sulfhydryl groups does not alone prove that this is the cause of the inactivation. Rigid proof that this is so may be provided by the demonstration that chemical blocking and protection of sulfhydryl groups results in protection of the enzyme function. Attempts to carry out such experiments were, however, unsuccessful since it was not possible to reactivate the enzyme in a reproducible manner by removal of the sulfhydryl blocking agent after the irradiation. Further evidence for the role of sulfhydryl group destruction in the X-ray inactivation was therefore sought in other types of experiment.

If the radiation-induced loss of an enzyme activity is caused by destruction of sulfhydryl groups directly involved in its function, the activity would be expected to be lost in an all-or-none fashion. This implies that enzyme molecules exposed to radiation should either possess full activity or have completely lost their activity. In order to ascertain whether this is so with the present enzyme, the effect of irradiation on the kinetic parameters of the dehydrogenase activity, as well as on the sensitivity of the dehydrogenase to threonine, was measured. It is apparent from Fig. 4A that the reduction in V occurring upon irradiation is associated with a

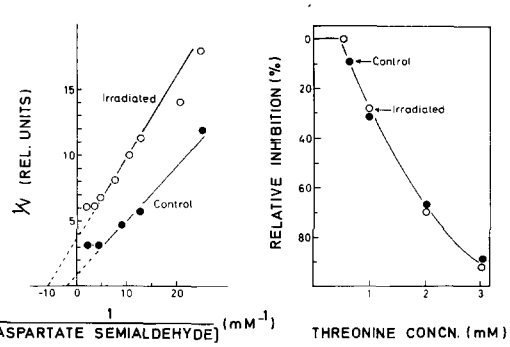

Fig. 4. Effect of irradiation on the kinetic parameters and on the sensitivity of the dehydrogenase activity to threonine. The enzyme ($0.50 \cdot 10^{-6}$ M) was irradiated with a dose of 30 kR resulting in a decrease in the catalytic activity of 40% and in the sensitivity to threonine of 60%. A. Inverse plot of dehydrogenase activity *versus* substrate concentration in native and irradiated enzyme. B. Inhibition of the enzyme by threonine as a function of its concentration. Both for the native and the irradiated enzyme the inhibition obtained in the presence of 10 mM threonine was set equal to 100%.

decrease in the K_m for the substrate, indicating that irradiation leads to the formation of enzyme molecules that are partly inactivated. The data indicate that the destruction of the sulfhydryl groups is not directly involved in the loss of the dehydrogenase activity.

The effect of irradiation on the sensitivity of the dehydrogenase to threonine is shown in Fig. 4B. Both for the native and the irradiated enzyme the inhibition observed in the presence of a large excess (10 mM) of threonine was set equal to 100. When the results are normalized and the relative inhibition expressed as a function of the threonine concentration, the curves for the native and the irradiated enzyme coincide. This indicates that the binding constant of threonine is unchanged upon irradiation and that the irradiated enzyme consists of a mixture of fully sensitive

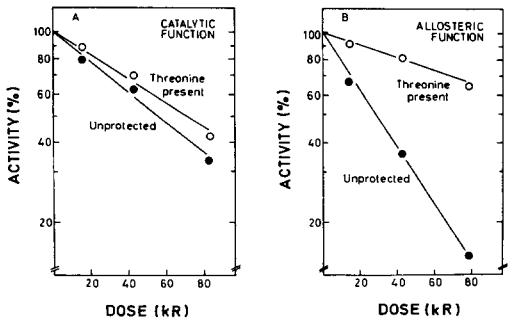

Fig. 5. Preferential protection of the allosteric function of the dehydrogenase activity by threonine. The enzyme ($0.50 \cdot 10^{-6}$ M) was irradiated in the absence and presence of 2 mM DL-threonine. The catalytic and allosteric activities were determined as previously described.

and completely desensitized molecules. The data are consistent with the view that the radiation-induced loss of the allosteric dehydrogenase function involves the destruction of sulfhydryl groups.

Previous studies[7,8] have shown that the presence of threonine protects the enzymes sulfhydryl groups. The results in Fig. 5A show that the presence of 2 mM threonine during irradiation had only a slight protective effect on the catalytic dehydrogenase activity, while the allosteric function was protected by a dose reduction factor of more than 5 (Fig. 5B). Thus, the allosteric effector preferentially protects the allosteric sensitivity of the dehydrogenase against X-ray inactivation.

DISCUSSION

The present results show that the different functions of this complex enzyme exhibit different sensitivities to ionizing radiation. The allosteric function of the dehydrogenase activity was, as in several other allosteric enzymes[17–19], considerably more sensitive to radiation than the catalytic function.

X-rays may inactivate enzymes by destroying specific active groups, by altering the conformation of the protein, or by a combination of these factors. Previous studies of sulfhydryl enzymes have shown that in all instances the radiation inactivation in dilute solution could be accounted for largely by destruction of sulfhydryl groups[20,21]. The present results indicate that sulfhydryl group destruction also plays an important role in this case. Thus, the data provide strong evidence that the X-ray-induced loss of the sensitivity of the dehydrogenase to threonine can be satisfactorily accounted for by destruction of sulfhydryl groups. Firstly, the X-ray destruction of sulfhydryl groups, compared with the effect of chemical sulfhydryl blocking, was shown to be sufficiently large to be able to account for the inactivation. Secondly, the presence of threonine, which is known to protect the enzyme sulfhydryl groups toward chemical reagents[7,8], strongly protected the allosteric dehydrogenase function against X-rays, but had little or no protective effect on the catalytic activity. Furthermore, kinetic experiments indicated that the loss of the allosteric dehydrogenase function is an all-or-none process, as would be expected were it caused by destruction of specific chemical groups.

The present finding that the catalytic kinase activity and the allosteric

dehydrogenase activity were destroyed by X-rays at exactly the same rate also suggests that the loss of the kinase activity after irradiation was due to destruction of sulfhydryl groups. This follows from the fact that the two enzyme functions are equally sensitive to sulfhydryl blocking agents[8] and the finding that the catalytic kinase activity can be completely abolished by DTNB. The present finding that the sensitivity of the kinase to threonine was unaffected by irradiation is consistent with the observation that during sulfhydryl group titration the remaining kinase activity retains its sensitivity to inhibition by threonine[8].

BARBER AND BRIGHT[9] have observed that sulfhydryl blocking by *p*-chloromercuribenzoate results in dissociation of the native enzyme into smaller species possessing dehydrogenase activity. Concomitantly with the dissociation the ability of the enzyme to bind threonine is lost. On this basis it appears likely that oxidation of sulfhydryl groups by X-rays results in dissociation of the enzyme with consequent loss of the dehydrogenase inhibition by threonine, and loss of kinase activity. The fact that the sensitivity of the kinase to threonine is unaffected by sulfhydryl blocking agents or by X-rays can readily be explained by assuming that the catalytic kinase activity and the ability of the enzyme to bind threonine are lost concomitantly. Consequently, enzyme molecules that have retained catalytic kinase activity have also retained their full sensitivity to threonine.

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