BBA 65861

X-RAY MODIFICATION OF THE CATALYTIC AND ALLOSTERIC FUNCTIONS OF FRUCTOSE-1,6-DIPHOSPHATASE

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(Received September 27th, 1968)

SUMMARY

1. Native rabbit liver fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) and enzyme stimulated by disulfide formation have been irradiated in solution with X-rays.

2. The dose–response curve for the native enzyme showed an initial stimulation of catalytic activity, followed by subsequent exponential inactivation. The disulfide-modified enzyme was inactivated exponentially with no initial stimulation. The G values for inactivation of catalytic activity were 0.017 and 0.009 for the native and the disulfide-modified enzymes, respectively.

3. The allosteric activity of the native enzyme, as measured by the inhibition by AMP, was much more radioresistant than the catalytic activity. In the modified enzyme, these two functions were of comparable radiosensitivity.

4. The ability of the enzymes to be stimulated by mixed disulfide formation was about 10 times as sensitive to X-ray inactivation as was the catalytic function. The loss of sensitivity to stimulation by disulfides was due to destruction of 5–6 specific SH groups which were particularly radiosensitive (*G* value, 1.2).

5. The X-ray-induced reduction in enzyme activity was associated with destruction of enzyme SH groups of low radiosensitivity (*G* value, 0.07). The loss of enzyme activity was partly reversed by addition of cysteamine after exposure. The results indicate that sulfhydryl groups play an important, but not exclusive role in the X-ray inactivation of fructose-1,6-diphosphatase.

6. The presence of AMP during irradiation protected the enzyme almost completely against inactivation of the allosteric function. The results indicated that

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTNB-enzyme, fructose-1,6-diphosphatase where 5–6 SH groups have been blocked by DTNB; PCMB-enzyme, fructose-1,6-diphosphatase where 5–6 SH groups have been blocked by PCMB.

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the X-ray inactivation of the allosteric function of fructose-1,6-diphosphatase is due to a specific modification of the binding sites for AMP.

INTRODUCTION

Allosteric enzymes are assumed to play an important role in the control of cellular metabolism. The possibility has therefore been considered[1,2] that radiation damage of the allosteric function of such enzymes may play a greater role in cellular radiation damage than loss of their catalytic activity. With two allosteric enzymes previously studied, aspartate transcarbamylase, and phosphorylase *b*, the allosteric function proved to be more sensitive to radiation damage than the catalytic function[1–3]. In the case of phosphofructokinase more complex relationships have been found[4].

Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) is an allosteric enzyme which plays an important role in the control of gluconeogenesis. Fructose-1,6-diphosphatase is inhibited by the allosteric modifier AMP (refs. 5 and 6) and the enzyme shows a complex response to modification of its sulfhydryl groups. Thus, blocking of up to 5–6 SH groups stimulates the enzyme, while further blocking causes progressive loss of activity[7–9]. The allosteric function of the enzyme shows a similar response to sulfhydryl modification[9].

Since fructose-1,6-diphosphatase, as isolated, shows little activity at neutral pH, it has been suggested that mixed disulfide formation with physiologically occurring disulfides may play an important role in the regulatory function of the enzyme[8]. This view prompted us to compare the effects of X-rays on native fructose-1,6-diphosphatase and fructose-1,6-diphosphatase stimulated by mixed disulfide formation. One of the main objectives was to assess the relative radiation sensitivity of the catalytic and the allosteric functions in the native and the stimulated enzyme.

MATERIALS AND METHODS

Materials

FDP, AMP, NADP, cysteamine hydrochloride, 5,5′-dithio-bis(2-nitrobenzoic acid)-(DTNB), *p*-chloromercuribenzoate (PCMB), and glucose-6-phosphate isomerase were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose 6-phosphate dehydrogenase was supplied by Boehringer and Soehne, Mannheim, Germany.

Preparation of enzyme

The enzyme was prepared from rabbit liver as described by PONTREMOLI *et al.*[10]. During the preparation the enzyme was passed only once through an ion-exchange column. Volume reduction was carried out by a Sephadex G-25 swelling technique[11].

Sulfhydryl assays were carried out by the methods of ELLMAN[12] and of BOYER[13] on the enzyme denatured in 8 M urea.

Protein concentration was determined by measuring the absorbance of the enzyme solution at 260 and 280 mμ[10] and further checked using the protein determination of LOWRY *et al.*[14]. A mol. wt. of 127 000 was used[7].

Enzyme assay

Enzyme activity was measured by the method of PONTREMOLI *et al.*[10]. The enzyme is coupled *via* glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase to NADP and the rate of NADP reduction is measured spectrophotometrically at 340 mμ. The assay mixture contained 0.2 mM FDP, 0.1 M glycine buffer, pH 9.4, 1 mM $MnCl_2$, together with a large excess of glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase and NADP. The reaction was carried out at room temperature (23–25°) in a vol. of 1 ml. One unit of enzyme activity is defined as that amount of enzyme which causes an absorbance change (light path, 1 cm) of one $A_{340\ m\mu}$ unit per min under the above conditions. The enzyme preparations used had a specific activity of at least 120 units/mg.

Preparation of DTNB-enzyme

4 μM fructose-1,6-diphosphatase in 20 mM Tris buffer (pH 7.5) was incubated with a 500-fold molar excess of DTNB for 30 min. In order to remove unbound DTNB the incubation mixture was then applied to a Sephadex G-25 column and eluted using 5 mM malonate buffer (pH 6.8). Eluted fractions containing enzyme activity were combined, and if necessary, were concentrated by G-25 swelling.

Irradiation conditions

The enzyme was irradiated in flat-bottomed tubes in the presence of air at 0°. The irradiation source was a Stabilipan X-ray machine. The irradiation parameters were 220 kV, 20 mA with 0.5-mm copper filtration. The dose rate, measured with a Fricke dosimeter, was 1.9 kR/min.

Unless otherwise stated, all reactions were carried out at 23–25°.

RESULTS

Effect of X-rays on the catalytic function

The activity of fructose-1,6-diphosphatase can be stimulated by modification of some of its sulfhydryl groups, as pointed out above. Per sulfhydryl group modified, disulfides are more effective in stimulating the enzyme than are other SH reagents, and can cause up to 7-fold stimulation[8,9]. When more than 5–6 SH groups are blocked, the activity again decreases[7,9]. The available data indicate that the stimulation of fructose-1,6-diphosphatase by modification of SH groups is due to concomitant conformational changes of the enzyme[9]. The results in Fig. 1A show that the native enzyme and enzyme where 5–6 SH groups had been blocked by mixed disulfide formation with DTNB (here denoted DTNB-enzyme) exhibited different dose–response curves. While the DTNB-enzyme gave a linear dose–response curve in a semilogarithmic plot, the native enzyme showed a small but definite stimulation by small doses of radiation and was subsequently inhibited by greater doses. The initial stimulation was found to be dependent on the pH during the irradiation. Thus, the stimulation was 5 to 6 times greater when the enzyme was irradiated at pH 9 than at pH 4.

The yields of inactivation for the native and the DTNB-enzyme were calculated from the lines in Fig. 1B where the D_{37} doses derived from the exponential part of the dose–response curves are plotted against the enzyme concentration. The G values

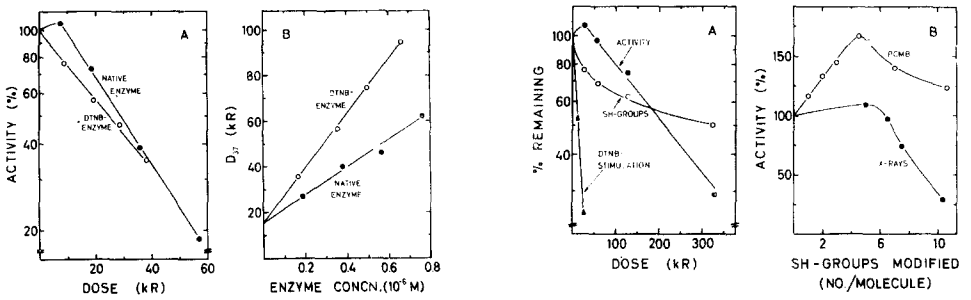

Fig. 1. X-ray inactivation of fructose-1,6-diphosphatase. Native enzyme (0.19 μM) and DTNB-enzyme (0.17 μM) in 2 mM malonate buffer (pH 6.8) were irradiated in equilibrium with air at 0°. A. Dose–response curves. The activities expressed in % of that of the unirradiated controls. B. The D_{37} dose as a function of the enzyme concentration. The D_{37} doses were obtained from the exponential part of dose–inactivation curves, measured under the above conditions.

Fig. 2. Disappearance of SH groups of native fructose-1,6-diphosphatase upon irradiation. Fructose-1,6-diphosphatase (2.5 μM) in 5 mM malonate buffer (pH 6) was irradiated with increasing doses of X-rays. A. Dose–response curves for SH disappearance, enzyme activity and stimulation by DTNB. All values are expressed in % of those of the unirradiated controls. The native enzyme contained 20 SH groups per molecule. B. Relationship between enzyme activity and number of SH groups modified. The data for X-rays are taken from Fig. 2A, while the data for PCMB are taken from LITTLE, SANNER AND PIHL[9].

(the number of enzyme molecules inactivated per 100 eV absorbed by the solution) were 0.017 for the native and 0.009 for the DTNB-enzyme. Thus, the modified enzyme was considerably more resistant to irradiation then the native enzyme.

Role of sulfhydryl groups

Fructose-1,6-diphosphatase contains 5–6 particularly reactive SH groups, the modification of which leads to stimulation of the activity[9]. The fact that irradiation of the native enzyme resulted in an initial stimulation, while no such stimulation occurred in the DTNB-enzyme, suggests that the radiation-induced stimulation of fructose-1,6-diphosphatase involves modification of these reactive SH groups. In order to study this in more detail the loss of sulfhydryl groups on irradiation was measured and related to the concurrent changes in enzyme activity. The results in Fig. 2A demonstrate that irradiation of the native enzyme leads to a rapid initial loss of SH groups, followed by a slower rate of SH destruction. The shape of the response curve indicates that the enzyme contains sulfhydryl groups of different radiation susceptibility. Extrapolation of the final slope of the curve to zero dose indicates that 5–6 sulfhydryl groups are more susceptible to X-rays than are the remainder. It is seen that the more sensitive SH groups disappear in the dose range where stimulation of enzyme activity occurs. Concurrently with the loss of these SH groups the enzyme also loses its ability to become stimulated by DTNB. Altogether these results provide strong evidence that the 5–6 SH groups which are sufficiently reactive to be blocked by DTNB, are particularly susceptible to destruction by ionizing radiation, and that the radiation-induced stimulation of the enzyme activity is due to destruction of these SH groups. The finding that a correlation exists between the reactivity of SH groups towards chemical reagents and their susceptibility to ionizing radiation is in agreement with our previous findings[15,16].

The relationship between modification of enzyme SH groups and change in catalytic activity is shown in Fig. 2B. For comparison, similar data are included for PCMB-treated enzyme. It can be seen that, both after X-rays and after PCMB treatment, the enzyme activity increased when up to 5 sulfhydryl groups were altered, and subsequently decreased when additional SH groups were modified. Interestingly, per SH group modified, PCMB was far more effective in stimulating the enzyme than were X-rays. In contrast, the decreasing part of the curves demonstrates that, per SH group modified, X-rays inactivated the enzyme more effectively than did PCMB.

In Fig. 3 analogous data are given for the DTNB-enzyme. In this case, the sulfhydryl groups disappeared as a linear function of the radiation dose, in contrast to the results with the native enzyme. This was expected as the more radiosensitive sulfhydryl groups revealed in Fig. 2A had already been blocked by DTNB. It is

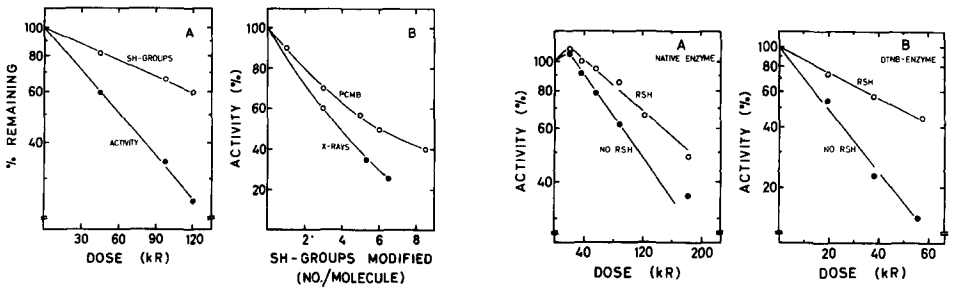

Fig. 3. Disappearance of SH groups in DTNB-enzyme upon irradiation. DTNB-enzyme (0.6 μM) in 5 mM malonate buffer (pH 6) was irradiated with increasing doses of X-rays. A. Dose–response curve for SH disappearance and enzyme activity. B. Relationship between enzyme activity and number of SH groups modified. The data for X-rays are taken from Fig. 3A, while the data for PCMB are taken from LITTLE, SANNER AND PIHL[9].

Fig. 4. The reactivating effect of cysteamine on irradiated fructose-1,6-diphosphatase. Native fructose-1,6-diphosphatase (2 μM) and DTNB-enzyme (0.2 μM) were irradiated in 2 mM malonate buffer (pH 6.8). Immediately after exposure the samples were incubated at pH 7.5 for 45 min, with or without 1 mM cysteamine, and the activities were measured. A. Native enzyme. B. DTNB-enzyme.

apparent from Fig. 3B that also in this case X-rays inactivated the enzyme more effectively per SH group modified, than did PCMB. Since X-rays probably react less selectively with SH groups of different reactivity than does PCMB, these data indicate that the X-ray inactivation of fructose-1,6-diphosphatase cannot be entirely accounted for by destruction of SH groups but may in part be due to modification of other residues.

In previous studies on sulfhydryl enzymes it has been found that in certain cases, the radiation inactivation could be partly reversed by the addition of thiols after exposure[15,17]. It was therefore of interest to see whether this was the case also with fructose-1,6-diphosphatase. It is apparent from Fig. 4 that the X-ray inactivation was less when cysteamine was added after the irradiation. This effect was more pronounced in the DTNB-enzyme than in the native enzyme. The results support the view that oxidation of sulfhydryl groups is involved in the X-ray inactivation of fructose-1,6-diphosphatase.

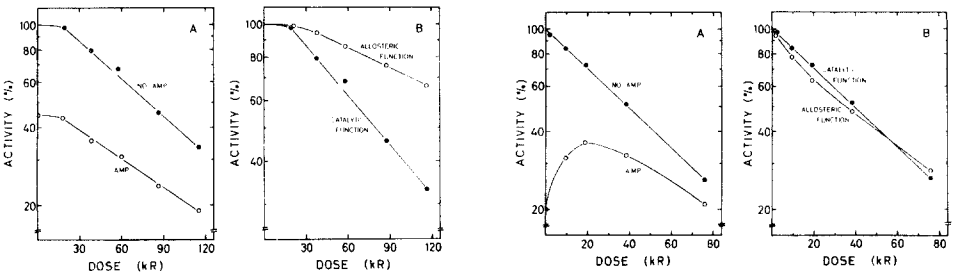

Fig. 5. Effect of X-rays on the allosteric function of fructose-1,6-diphosphatase. Fructose-1,6-diphosphatase (1.5 μM) in 5 mM malonate buffer (pH 6.8) was irradiated. A. Dose–response curve for enzyme activity, assayed in the presence and absence of 0.5 mM AMP. B. Disappearance of the catalytic and allosteric function. The allosteric function was calculated as the degree of inhibition by AMP, expressed in % of the inhibition of the unirradiated enzyme.

Fig. 6. Effect of X-rays on the allosteric function of DTNB-enzyme. The enzyme concentration was 0.4 μM. Other conditions and notation are as in Fig. 5.

Effects of X-rays on the allosteric function

Fructose-1,6-diphosphatase is inhibited by the allosteric modifier AMP and by high concentrations of substrate[5,6]. In Fig. 5A is shown the radiation-induced loss of enzyme activity, measured in the presence and absence of AMP. It can be seen, that, in the presence of AMP, the activity decreased, after an initial threshold dose, as an exponential function of the dose. The slope of the exponential part of the curve was less than that observed in the absence of AMP. Consequently, with increasing doses the curves approached each other, implying that, concurrent with the loss of catalytic activity, the allosteric activity was reduced. In order to compare the radiation sensitivity of the two functions, both were expressed in per cent of that of the unirradiated control (Fig. 5B). The data demonstrate that in this case the allosteric function is far more resistant to irradiation than is the catalytic function.

In Fig. 6 similar data are shown for the DTNB-enzyme. At zero dose the inhibition by AMP was considerably greater than in the native enzyme (Fig. 5) in spite of the fact that the same AMP concentration was used, in agreement with our previous findings[9]. It is apparent from Fig. 6A that the activity measured in the presence of AMP showed an initial increase, indicating that the allosteric activity was more rapidly lost upon irradiation than was the case in the native enzyme. This is confirmed in Fig. 6B which shows that in the DTNB-enzyme, the allosteric function is at least as sensitive to irradiation as is the catalytic function.

One obvious explanation for the radiation-induced loss of allosteric activity is that the ability of the enzyme to bind AMP might be lost upon irradiation. In order to test this possibility the inhibiting effect of increasing AMP concentrations on the unirradiated and irradiated enzymes was measured. In Fig. 7A the results for the DTNB-enzyme are shown. It is seen that after a radiation dose giving 50% inactivation, the inhibition of the enzyme by AMP was considerably reduced at all AMP concentrations. The shape of the curve suggested that irradiation reduced the binding constant for AMP. In Fig. 7B double-reciprocal plots are shown for the data in Fig. 7A, as well as for similar data obtained with native fructose-1,6-diphosphatase. It is seen that for AMP concentrations greater than 0.5 mM, nearly linear curves

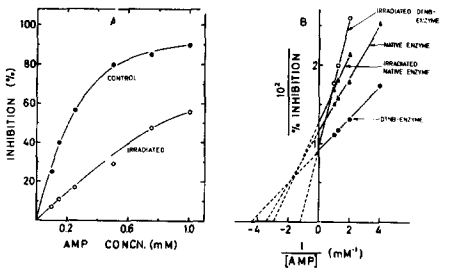

Fig. 7. Effect of irradiation on AMP inhibition of fructose-1,6-diphosphatase. Enzyme (0.9 μM) in 5 mM malonate buffer (pH 6.8) was irradiated until 50% of the activity remained. The activity was assayed in the presence of increasing concentrations of AMP. A. Inhibition of DTNB-enzyme as a function of AMP concentration. B. Double-reciprocal plots of inhibition *versus* AMP concentration. The data for DTNB-enzyme are taken from Fig. 7A, while the other data are taken from analogous experiments with native fructose-1,6-diphosphatase.

were obtained. The intercept on the ordinate may be taken as a measure of the maximum degree of inhibition by AMP, and the intercept on the abscissa as a measure of the reciprocal of the dissociation constant of the enzyme for AMP. In the case of the DTNB-enzyme irradiation increased the apparent dissociation constant by about 600%, while the maximal inhibition seemed to be unaffected .In the native enzyme irradiation increased the dissociation constant by only about 30%, while concurrently the maximal degree of inhibition was slightly reduced. It thus appears that the strong effect of irradiation on the allosteric function of the DTNB-enzyme was due to the fact that irradiation strongly reduced the binding of AMP to this modified enzyme.

In experiments where the effect of X-rays on the substrate inhibition was studied, it was found that in the native, as well as in the DTNB-enzyme, irradiation reduced the inhibitory effect of substrate only slightly.

The effect of pH on the relative radiation sensitivity of the catalytic and the allosteric properties of fructose-1,6-diphosphatase is demonstrated in Table I. It can be seen that both functions seemed to become more radioresistant with increasing pH. This could be due to the fact that the ability of the glycine buffer to capture OH radicals increases strongly with increasing pH[18]. At all pH values studied the catalytic

TABLE I

EFFECT OF pH ON THE RELATIVE RADIATION SENSITIVITY OF THE CATALYTIC AND THE ALLOSTERIC FUNCTIONS

Native fructose-1,6-diphosphatase (0.4 μM) was irradiated with increasing doses of X-rays, in 25 mM glycine buffers at the pH's indicated, and the activity was assayed in the presence and absence of 0.25 mM AMP. The catalytic and allosteric activities were calculated and plotted as in Fig. 5B, and the D_{37} doses were derived from the exponential part of the curves.

pH	D_{37} *catalytic activity (kR)*	D_{37} *allosteric activity (kR)*	$\frac{D_{37}\ allosteric\ activity}{D_{37}\ catalytic\ activity}$
4.0	61	100	1.6
7.5	65	110	1.7
9.0	125	300	2.4

function was more sensitive to radiation than was the allosteric function. The relative sensitivity was approximately the same at pH 4 and 7.5 while at pH 9 the ratio was much greater. The latter effect of pH cannot be explained by changes in the radical scavenging ability of the buffer. Apparently the change in pH altered the sensitivity of the two sites to different extents. The results suggest that different residues are involved in the loss of activity of the two functions.

Further support for the view that different residues are involved in the loss of the catalytic and allosteric function was obtained in experiments where the enzyme was irradiated in the presence of the substrate (fructose 1,6-diphosphate) and the allosteric modifier (AMP). It was found (Table II) that the presence of AMP during

TABLE II

THE PROTECTIVE EFFECT OF SUBSTRATE AND AMP ON X-RAY INACTIVATION OF THE CATALYTIC AND ALLOSTERIC FUNCTIONS

Fructose-1,6-diphosphatase (2.8 μM) in 5 mM malonate buffer (pH 6) was irradiated with 190 kR in the presence and absence of substrate or AMP. The activity was assayed in the presence and absence of 0.5 mM AMP at a substrate concentration of 1.25 mM. The allosteric activity is expressed as in Fig. 5B.

Additions	*Inactivation (%)*		*Relative inactivation* $\left(\frac{\text{Catalytic function}}{\text{Allosteric function}}\right)$
	Catalytic function	*Allosteric function*	
None	79	63	1.3
AMP (1 mM)	68	8	8.5
Fructose 1,6-diphosphate (1 mM)	50	68	0.7

irradiation almost completely prevented the loss of allosteric function, while the loss of catalytic activity was only slightly reduced. On the other hand, the presence of 1 mM substrate afforded a definite but moderate protection of the catalytic activity, but offered no protection against the inactivation of the allosteric property of the enzyme. It is thus clear that the catalytic and allosteric functions could be rather specifically protected by the presence of compounds which bind to the respective sites. The results indicate that the radiation-induced loss of the allosteric function is due to rather specific damage of residues involved in the binding site for AMP. Also, in the case of the catalytic function, damage to residues involved in the binding of substrate is of importance. However, in this case damage to other residues also seems to be of importance for the activity.

DISCUSSION

The present results, as well as those recently obtained with phosphofructokinase[4], demonstrate that no generalization is warranted concerning the relative radiation sensitivity of the catalytic and allosteric sites in regulatory enzymes. Thus, in fructose-1,6-diphosphatase, as isolated, the allosteric function proved to be much less susceptible to radiation than the catalytic function. It is noteworthy, however, that in fructose-1,6-diphosphatase which had been stimulated by mixed disulfide

formation with DTNB, the allosteric function was more radiosensitive and the catalytic function more radioresistant than in the native enzyme. The results indicate that in disulfide-modified enzymes, which may be the physiologically active forms, the two enzymic functions may have about equal radiation sensitivity.

Probably the most interesting observation from a physiological point of view is the finding that small doses of X-rays, which hardly affected the catalytic activity of the enzyme, almost abolished the sensitivity of the enzyme to stimulation by mixed disulfide formation (Fig. 2A). Thus, the sensitivity of the enzyme to stimulation by DTNB was destroyed with a yield that was more than 10 times as great as that for the loss of catalytic activity. If disulfide modification of fructose-1,6-diphosphatase is indeed important in the regulatory function of the enzyme, as suggested by PONTREMOLI *et al.*[8], this implies that the regulatory function of fructose-1,6-diphosphatase is in fact much more sensitive to radiation than is the catalytic function. The reason for the rapid loss of ability of the enzyme to be stimulated by disulfides is that this stimulation is associated with 5–6 SH groups, which are particularly reactive, and are preferentially destroyed by ionizing radiation. Thus, the titration of the disappearance of SH groups upon irradiation provided direct evidence that 5–6 SH groups had a much higher radiosensitivity than the remainder. This preferential destruction of the SH groups involved in stimulation of the native enzyme also explains the complex dose–effect curve observed. The initial stimulation of the activity observed upon irradiation was undoubtedly due to modification of these special SH groups.

The loss of catalytic activity upon irradiation was associated with destruction of the remaining, less reactive sulfhydryl groups, suggesting that these play a role in the inactivation of the enzyme. Blocking of these SH groups by PCMB would therefore be expected to protect the enzyme against X-ray inactivation. Unfortunately, such experiments could not be carried out since blocking of these sulfhydryl groups with PCMB caused irreversible inactivation of the enzyme. However, the significance of these sulfhydryl groups in X-ray inactivation of fructose-1,6-diphosphatase was further indicated by the finding that the inactivation could be partly reversed by addition of cysteamine after exposure. The fact that X-rays were more effective, per SH group modified, in inactivating the enzyme than PCMB indicated that the enzyme inactivation could not be entirely accounted for by destruction of sulfhydryl groups, and that other residues probably are involved in the inactivation.

The data presented indicate that mixed disulfide formation of the SH groups of fructose-1,6-diphosphatase with DTNB provides considerable protection against radiation-induced loss of catalytic activity. This follows from the data given in Fig. 1B and Fig. 4. This protection by mixed disulfide formation requires some comment. Previously we have shown[15,19] that in cases where sulfhydryl groups are essential for enzymatic activity, the blocking of such groups by mixed disulfide formation will afford considerable protection against radiation inactivation. Such a mechanism, however, cannot account for the protection of fructose-1,6-diphosphatase by DTNB treatment, since in this case destruction of the SH groups modified does not lead to loss of enzyme activity. Presumably, in the case of fructose-1,6-diphosphatase the protective effect is due to conformational changes associated with the DTNB blocking. Evidence that mixed disulfide formation on fructose-1,6-diphosphatase leads to conformational changes has previously been presented[9].

The fact that the allosteric site could be effectively and specifically protected by carrying out the irradiation in the presence of AMP indicates that the inactivation of the allosteric function is primarily due to modification of the allosteric sites as such, and not the result of damage to other parts of the molecule. Previous results indicate that tyrosine groups are involved in the allosteric sites of fructose-1,6-diphosphatase[9,20]. Since tyrosine belongs to the amino acids which are most extensively destroyed when proteins are irradiated in solution[21] it seems reasonable to assume that X-ray damage to tyrosine residues may in part account for the inactivation of the allosteric function of fructose-1,6-diphosphatase.

ACKNOWLEDGEMENT

This work was supported by the National Center for Radiological Health, Bureau of State Service, U.S. Public Health Service.

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