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X-RAY MODIFICATION OF THE ALLOSTERIC FUNCTIONS OF RAT THYMOCYTE PHOSPHOFRUCTOKINASE*

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SUMMARY

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) was extracted and partially purified from rat thymocytes. The enzyme in solution was then irradiated with X-rays at doses ranging from 0.5 to 10 kR. The native rat thymocyte phosphofructokinase was strongly inhibited by ATP at a concentration equivalent to the intracellular level in thymocytes. X-ray irradiation brought about a dose-dependent decrease in the ATP inhibition. As a result, stimulation of phosphofructokinase activity took place under conditions comparable to those within the cell. The apparent K_m and the Hill coefficient of the enzyme were decreased by irradiation, resulting in enhancement of the enzyme activity. The possibility that the facilitation of phosphofructokinase reaction in irradiated thymocytes observed earlier might be due to a reduced sensitivity of the enzyme to ATP inhibition was discussed.

INTRODUCTION

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) represents an important control point in glycolysis in different mammalian tissue [1–3]. Earlier work from this laboratory[4–6] indicated that the enzyme was also one of the most important enzymes in the regulation of glycolysis in rat thymocytes, and its regulatory properties were profoundly affected by X-ray irradiation, that is, aerobic glycolysis was markedly stimulated by low dose X-ray irradiation as a result of enhancement of the phosphofructokinase reaction. Investigations of the X-ray effect on phosphofructokinase in solution, so far performed with enzyme preparations from muscle, have demonstrated a marked effect of X-rays on the catalytic functions of the enzyme[7,8].

In a preceding paper[9] we have shown that phosphofructokinase extracted and

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purified from rat thymocytes possesses stronger allosteric properties than the muscle enzyme. Thymocyte phosphofructokinase showed a higher susceptibility to ATP inhibition and a greater sensitivity to allosteric effectors than the muscle phosphofructokinase. Therefore, it is of considerable interest to examine the effects of X-ray irradiation on thymocyte phosphofructokinase in solution. Such a study might provide some clue for the elucidation of the mechanism underlying the enhancement of the phosphofructokinase reaction in irradiated rat thymocytes. In the present work we have examined the effect of X-rays on thymocyte phosphofructokinase in solution. Our results indicate a greater sensitivity of the allosteric function of the enzyme to X-rays, and, particularly, a reduction of the enzyme sensitivity to ATP inhibition leading to the enhancement of the phosphofructokinase reaction.

MATERIALS AND METHODS

Enzyme preparation

The enzyme was extracted and partially purified from the thymus tissues of the Wistar strain rats from our breeding colony as described previously[9], except that the final purification step of affinity chromatography on blue dextran polyacrylamide gel was omitted. The purification procedures therefore included $(NH_4)_2SO_4$ fractionation, heat treatment and TEAE-cellulose column chromatography. The enzyme preparation thus obtained had a specific activity of at least 5 units per mg protein. Units of activity were defined as the number of μmoles of fructose 1,6-diphosphate produced per min under the standard conditions[9]. The enzyme was diluted with a solution containing 25 mM Tris–HCl, pH 8.0, 25 mM $(NH_4)_2SO_4$, 1 mM EDTA, 0.1 mM mercaptoethanol, 0.1 mM ATP and 0.01 mM fructose 1,6-diphosphate. Prior to irradiation, mercaptoethanol was removed from the enzyme solution by passage through a Sephadex G-25 column, since the SH-reagent has a strong protective action against X-ray irradiation.

Irradiation conditions

The enzyme solution was then irradiated in a cylindrical polycarbonate tube in the presence of air at 0 °C with X-rays delivered at a rate of 360 R/min by a Shimazu radiotherapy-type machine operated at 200 kVp and 20 mA, with 0.5 mm Cu *plus* 0.5 mm Al filters (h.v.l., 1.2 mm Cu).

Enzyme assay

Immediately after irradiation, enzyme assays and kinetic experiments were carried out under the conditions described previously[9]. Enzyme activity was determined at pH 7.2 by spectrophotometrically measuring the rate of NADH oxidation in a solution where the enzyme was coupled with aldolase (EC 4.2.1.13), triosephosphate isomerase (EC 5.3.1.1) and α-glycerophosphate dehydrogenase (EC 1.1.1.8). The details of this system were described in the preceding paper[9]. The coupling enzymes were free from $(NH_4)_2SO_4$ (ref. 9). The phosphofructokinase concentration per assay was about 0.01 unit per ml[9].

The chemicals used in this study were obtained from the sources given previously[9]. ATP was purchased from Kojin Co. Ltd, Tokyo, and purified by DEAE-cellulose chromatography as described in the preceding paper[9].

RESULTS

Reduced sensitivity of the rat thymocyte phosphofructokinase to ATP inhibition

Phosphofructokinase from rat thymocytes is strongly inhibited by the allosteric modifier ATP[9]. In Fig. 1 the radiation-induced reduction of the enzyme sensitivity to ATP inhibition measured at pH 7.2 in the presence of 0.1 mM fructose 6-phosphate is shown. It can be seen in this figure that the activity of the enzyme at a low, non-inhibitory concentration of ATP was essentially unaffected by irradiation. In contrast, the activity at an inhibitory concentration of ATP, especially at 1 mM ATP,

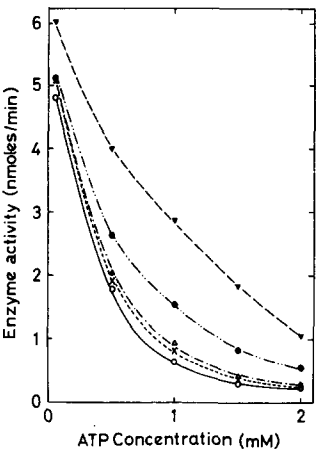

Fig. 1. Decrease in ATP inhibition of rat thymocyte phosphofructokinase by X-ray irradiation. The enzyme solution (1.2 units/ml) prepared from rat thymocytes was irradiated with 0.5–10 kR X-rays. The detailed procedures for the enzyme preparation and irradiation are described in the text. Immediately after irradiation, the ATP inhibition of the enzyme was measured under the conditions described in the text. ○—○, 0 kR; ×---×, 0.5 kR; △-·-△, 1 kR; ●-·-●, 5 kR; ▼---▼, 10 kR.

increased with increasing doses of X-rays. Even after lower doses the activity increase following X-ray irradiation was apparent: the enzyme activity after 0.5 kR–1 kR X-rays was 30–45% higher than that of the unirradiated control. Irradiation with higher doses of X-rays (5 kR and 10 kR) resulted in a 2–5-fold increase above that of the control at 1 mM ATP. These results indicate that irradiation with X-rays caused a marked decrease in ATP inhibition of the enzyme.

When the enzyme activity was assayed under the standard conditions[9] (pH 8.0, 1 mM ATP, 1 mM fructose 6-phosphate), no effect of X-ray irradiation could be observed (Table I). It was shown in the preceding investigation[9] that cyclic 3′,5′-AMP could completely reverse the ATP inhibition. X-ray irradiation with 5 kR exerted no significant, or rather slightly inhibitory, effect on the phosphofructokinase activity which was fully activated by the addition of 0.1 mM cyclic 3′,5′-AMP (Table II). These results suggest that X-rays within the dose range used might not inactivate the catalytic activity of the phosphofructokinase from rat thymocytes, whereas, as shown above, irradiation profoundly affected allosteric activity such as the sensitivity to ATP inhibition.

TABLE I

EFFECTS OF X-RAY IRRADIATION ON THE CATALYTIC ACTIVITY OF RAT THYMOCYTE PHOSPHOFRUCTOKINASE MEASURED IN THE PRESENCE OF 1 mM ATP AND 1 mM FRUCTOSE 6-PHOSPHATE AT pH 8.0

The enzyme activity was measured under the conditions mentioned in the text immediately after irradiation of the enzyme solution (1.2 units/ml). The other assay conditions, extraction and irradiation procedures are described in the text. Enzyme activity is expressed as nmoles of fructose 1,6-diphosphate produced per min under the conditions used.

Radiation dose (kR)	*Enzyme activity (nmoles/min)*
0	11.8
0.5	12.2
1	10.3
5	11.9
10	10.9

TABLE II

EFFECT OF 5 kR X-RAY IRRADIATION ON THE CATALYTIC ACTIVITY OF RAT THYMOCYTE PHOSPHOFRUCTOKINASE IN THE CYCLIC AMP-ACTIVATED STATE

Enzyme activity was measured in the presence of 0.1 mM fructose 6-phosphate and 0.1 mM cyclic AMP at pH 7.2. Enzyme activity was expressed as nmoles of fructose 1.6-diphosphate produced per min under the conditions used.

ATP concn (mM)	*Enzyme activity (nmoles/min)*			
	Without cyclic AMP		*With cyclic AMP*	
	Non-irradiated	*5 kR Irradiated*	*Non-irradiated*	*5 kR Irradiated*
0.05	4.82	5.15	9.19	8.80
1	0.64	1.56	9.00	8.20

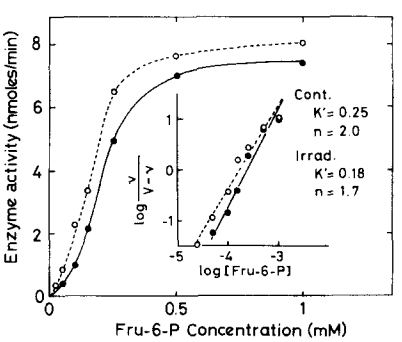

Fig. 2. Effect of 5 kR X-ray irradiation on fructose 6-phosphate saturation curves. Hill plots for rate *versus* fructose 6-phosphate concentration for native and irradiated phosphofructokinase are also shown in the figure. The enzyme in solution was irradiated at a concentration of 1.2 units/ml. Enzyme activity was measured in the presence of 1 mM ATP at pH 7.2. The details of the enzyme preparation, irradiation procedures and other assay conditions are described in the text. The enzyme activity was expressed as nmoles of fructose 1,6-diphosphate produced per min under the conditions used. ●—●, non-irradiated; ○---○, 5 kR irradiated; K', concentration of fructose 6-phosphate (mM) required to give half-maximal velocity (apparent K_m); n, Hill (interaction) coefficient.

Effect of X-ray irradiation on kinetics for fructose 6-phosphate

When the activity was determined at a given level of ATP (1 mM) with varying levels of fructose 6-phosphate, a sigmoidal response curve typical of allosteric enzymes was observed with the non-irradiated enzyme (Fig. 2). A Hill plot[10] of such data gave straight lines from which the concentration of substrate required to give half-maximal velocity (apparent K_m or K') and the Hill coefficient[10] (n) could be calculated. By X-ray irradiation with 5 kR, the saturation curve approached more closely a hyperbola, and the apparent K_m was lowered from 0.25 to 0.18 mM. The Hill coefficient (n) was slightly decreased after irradiation. These results might indicate that, by irradiation of the enzyme in solution with 5 kR, the affinity of the enzyme for fructose 6-phosphate was increased.

DISCUSSION

Investigations of X-ray effects on phosphofructokinase have previously been performed with the muscle enzyme[7,8]. Chapman *et al.*[7] have shown that X-ray irradiation induced a loss of the catalytic activity of phosphofructokinase prepared from rabbit muscle. The allosteric activity, as measured by the ability of AMP to reverse the ATP inhibition of the enzyme, was relatively resistant to radiation inactivation. Recently, Chumachenmko *et al.*[8] have reported an increased specific activity of phosphofructokinase in muscle homogenate from an irradiated rabbit. From the radiobiological point of view, an investigation on thymocytes is of particular interest because of their higher radiosensitivity[11]. For example, glycolysis in rat thymocytes is markedly stimulated by low doses of X-rays[4,12], whereas no effect could be detected on muscle glycolysis by such doses[13]. The results of the preceding investigation[9], which showed the stronger allosteric properties of the thymocyte phosphofructokinase, would lead us to expect that the effect of X-rays on thymocyte phosphofructokinase might be more profound than observed with the muscle enzyme.

The present data did indeed demonstrate the marked action of X-rays on the allosteric and kinetic properties of the thymocyte phosphofructokinase under conditions comparable to those within the cell. The X-ray irradiation within the dose range of 0.5–10 kR caused a marked decrease in the ATP inhibition of enzyme activity (Fig. 1). In particular, a marked stimulation could be observed at a concentration of ATP higher than 1 mM. The physiological (intracellular) concentration of ATP in the thymocytes is within the range of 1–3 mM[4,6]. When compared with the data for muscle phosphofructokinase[7], it can be noted that the ATP inhibition of thymocyte phosphofructokinase was more sensitive to X-ray irradiation. Although this difference in sensitivity to X-rays between the thymocyte and the muscle enzyme cannot yet be fully explained, this might be related to the difference in the allosteric properties of the enzymes[9].

A further interesting point which can be seen in Fig. 1 is the dose dependency of the X-ray-induced stimulation of enzyme activity. The enhancement of the enzyme activity increased with increasing doses. In a previous investigation[5] we demonstrated a dose dependent accumulation of fructose 1,6-diphosphate following exposure to the same radiation dose range. An enhancement of phosphofructokinase reaction was suggested from the calculated mass–action ratio of the reaction[5]. The mechanisms underlying the radiation-induced facilitation of the phosphofructokinase reaction in

rat thymocytes can be considered in several ways as follows[14]: (1) The *de novo* synthesis of phosphofructokinase may be activated by irradiation. (2) The intracellular localization of the enzyme may be altered by irradiation, leading to the facilitation of the enzyme reaction. (3) Changes in the concentration of allosteric effectors of the enzyme may be induced by irradiation, resulting in the stimulation of the enzyme activity. (4) The allosteric properties of the phosphofructokinase could be changed by irradiation so as to enhance the enzyme activity. Possibilities 1 and 3 can be ruled out from the results of our earlier experiments[5,15] where the effect of an inhibitor of protein synthesis and the time course of concentration changes in allosteric effectors were examined in the irradiated thymocytes. The results of the present investigation strongly suggest, therefore, the last possibility. From the results of kinetic experiments (Fig. 2), it seems reasonable to deduce that some alterations in kinetic and allosteric properties of phosphofructokinase, such as the binding affinity for fructose 6-phosphate and the sensitivity to ATP inhibition, are induced by X-irradiation and result in a marked stimulation of phosphofructokinase activity under the intracellular conditions of the irradiated thymocytes.

Several authors[16,17] have also reported the desensitization of phosphofructokinase to ATP inhibition after some treatments. Ahlfors and Mansour[16] reported that sheep heart phosphofructokinase became insensitive to ATP inhibition and that the Hill coefficient decreased after photo-oxidation. Desensitization of yeast phosphofructokinase to ATP inhibition by treatment with trypsin was demonstrated by Salas *et al.*[17]. Whether or not a common mechanism connects the above-mentioned phenomena with the radiation-induced desensitization of rat thymocyte phosphofructokinase reported here has not yet been examined and remains to be investigated.

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