Activation of Myosin-ATPase by Irradiated Histidine

UENO***, Akiko

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ABSTRACT

Activation of myosin-ATPase by irradiated histidine was studied. The kinetic constant and the optimum pH for the activated enzyme were found to be different from those of non-activated enzyme. An activating agent was separated from the irradiated histidine solution, through a column chromatography on ion-exchange resin. From its chemical properties and spectral characteristics, this was first assumed to be hydroxyhistidine. However, a peroxide which is more effective in activating the enzyme was also obtained from histidine on irradiation. Oxygen is needed for the production of this peroxide. Based on the studies of its sulfhydryl reactivity and heat stability, it was concluded that the peroxide concerned was not hydrogen peroxide, but presumably hydroxyhydroperoxide of histidine. From comparative studies on the activating effect on myosin of irradiated histidine and other sulfhydryl reagents, it was confirmed that irradiated histidine attacks a sulfhydryl group which is concerned with the conformation of myosin molecules, leading to an activation of myosin-ATPase.

INTRODUCTION

In a previous paper*, we have shown that the activation of myosin-ATPase by irradiation is markedly enhanced by the presence of histidine in the myosin solution and that equally marked activation of ATPase is induced when non-irradiated myosin solution is incubated with irradiated histidine. Further, the activating effect of irradiated histidine is completely suppressed by the presence of cysteine in the

* This paper is based on work performed at the Zoological Institute, Faculty of Science, University of Tokyo, Tokyo.
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*** Division of Biology, National Institute of Radiological Sciences, Chiba.
incubation mixture.

On the other hand, since the work of Kielley and Bladley on the chemical modification of myosin by sulfhydryl reagents such as PCMB and NEM, many studies have been done on the activation of myosin-ATPase by sulfhydryl reagents. From the results of our experiments and from those of other workers, it has been suggested that some kind of sulfhydryl-reactive substance which is effective in activating the myosin-ATPase is formed from histidine by irradiation and that sulfhydryl groups in myosin might be involved in the activation phenomenon.

The present paper describes the properties of myosin-ATPase activated by irradiated histidine and the nature of the activating agent(s) formed from histidine by irradiation. Comparative studies on the activating effect of irradiated histidine and other sulfhydryl reagents provide further confirmatory evidence for the activation mechanism of myosin-ATPase by irradiated histidine.

**MATERIAL AND METHODS**

*Enzyme preparation:* Myosin-ATPase was prepared from rabbit muscle according to the method of Perry with some modifications given in the previous paper. The enzyme preparation was stored at 0°C and diluted before use with 0.6 M KCl to a desired concentration.

*Assay method for ATPase activity:* The assay of ATPase activity was carried out in the following reaction mixture, unless otherwise specified: 0.15 M KCl, 4 x 10^{-3} M CaCl₂, 3 x 10^{-3} M ATP, 5 x 10^{-2} M Tris-HCl buffer, pH 9.1 and myosin (0.05–0.1 mg protein/ml), in a final volume of 2.0 ml. After incubation at 37°C for 10 min., the amount of inorganic phosphate formed was determined by the method of Lohmann and Jendrassik.

*Protein determination:* The method of Lowry et al. was employed. The standard curve for protein determination was calibrated from nitrogen of the myosin preparation determined by micro-Kjeldahl method.

*Isolation of the activating agent:* Isolation of activating agent from irradiated histidine solution was carried out by using the column chromatographic technique for separation of basic amino acids. Five mM histidine in 0.6 M KCl was irradiated with X-rays and 1 ml of the sample was applied to a column of Amberlite IR 120 (Na⁺ type, ×8, 100–200 mesh, 9 mm x 12 mm). Stepwise elution was carried out using 0.2 M acetate buffer, pH 4.25 followed by 0.35 M acetate buffer, pH 5.0. The elution rate was maintained at 30 ml per hour and 3.0 ml of fractions were collected.

*Histidine determination:* The histidine content of each fraction was determined according to the method of Cocking and Yemm. The pH of the eluate (1.0 ml)

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*Abbreviation used:* PCMB, p-chloromercuribenzoate; NEM, N-ethylmaleimide; ATP, adenosinetriphosphate; DTNB, 5,5'-dithiobis, 2-nitrobenzoic acid; EDTA, ethylenediamine tetraacetic acid.
ACTIVATION OF MYOSIN-ATPASE BY IRRADIATED HISTIDINE

was adjusted to 5 with 1 N NaOH, when necessary, and to the solution were added 2 ml of 0.2 M acetate buffer, pH 5.0 and 1.2 ml of KCN-ninhydrin solution. The mixture was heated in a boiling water bath for 15 min. After cooling, the volume of mixture was brought to 5 ml with 60% ethanol. Optical density at 570 mμ was determined spectrophotometrically.

The imidazole group of histidine was determined by Pauli's reaction\(^1\). The mixture, containing 1 ml of the eluate, 0.4 ml of 1% sulfanilic acid in 10% HCl and 0.4 ml of 5% NaNO₂, was shaken vigorously and allowed to stand at room temperature for 30 min. After adding 1.2 ml of 20% Na₂CO₃, the mixture was again shaken for 10 sec. and 2 ml of ethanol was added. The intensity of color developed was measured with a spectrophotometer at 570 mμ.

Reactivity to Folin's reagent: The mixture containing 2.5 ml of the eluate, 3 ml of 4% Na₂CO₃ and 0.5 ml of Folin's reagent\(^2\) was incubated for 20 min. at 37°C. Reactivity was expressed as unit of optical density at 660 mμ.

Peroxide determination: The amount of peroxide in irradiated solution was measured by the method used for estimation of glucose with some modifications\(^3\). To 0.5 ml of the solution to be examined was added 2.5 ml of the solution containing 0.6 mg of horse-radish peroxidase and 0.16 mg of o-dianisidine hydrochloride in 0.1 M phosphate buffer, pH 7.0 and the mixture was incubated for 15 min. at 37°C. The color produced was stabilized by addition of 2.5 ml of 18 N H₂SO₄. The intensity of red color developed was measured as 530 mμ. The amount of peroxide was expressed as hydrogen peroxide equivalent.

Sulphydryl group determination: The amount of the sulphydryl group was determined by the following two methods: (a) by the spectrophotometric procedure of Boyer\(^4\) for the sulphydryl group of myosin; (b) by Ellman's DTNB method\(^5\) for the sulphydryl group of myosin and cysteine.

Irradiation: The irradiation of the myosin and histidine solution was carried out at 0°C with a 200 kV Toshiba X-ray apparatus, operating at 15 mA, filtered through 1.0 mm aluminium, with a dose rate of 400 R per min. Other conditions were the same as described previously\(^6\). A part of irradiation was performed by \(^60\)Co γ-ray source at a dose rate of 6.4 kR per min.

Chemicals: ATP, horse-radish peroxidase, o-dianisidine, histidine, tris-(hydroxymethyl) amino methane and cysteine were purchased from Sigma Chem. Co. The DTNB was the product of Aldrich Chem. Co.

RESULTS

Properties of the activated ATPase: As reported previously\(^7\), the activation of myosin-ATPase is produced by the addition of irradiated histidine to an unirradiated myosin solution. Therefore, it seems probable that some changes are taking place in the properties of the enzyme. As shown in Fig. 1-a, the optimum pH of the activated ATPase shifts from pH 6.2 to 7.0. The Lineweaver-Burk plots for the activated ATPase are shown in Fig. 1-b. The values for \(K_m\) and \(V_{max}\) calculated
from the figure are listed in Table 1. $K_m$ value shows a slight decrease, while $V_{\text{max}}$ value increases to four-fold of that of the activated enzyme.

**Nature of the activating agent:** In order to know the nature of the activating agent, its isolation from the irradiated histidine has been attempted. Irradiated histidine solution was subjected to ion-exchange column chromatography as described in "Methods". Each eluate was analyzed for ninhydrin reactive substance (histidine and its derivatives), and also for the presence of activating agent on ATPase. Fig. 2 shows a typical result of the experiment. As can be seen from the figure, a peak of fractions having the activating effect on ATPase (first small peak) is well separated from the second peak of unknown nature and from the unchanged histidine fractions (third large peak). It was also found that the fractions having the activating effect react with sulfhydryl groups.

The histidine equivalent of the first small peak was calculated to be about 1 to 2% of the original histidine solution. Unlike histidine itself, the first small

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (M/L)</th>
<th>$V_{\text{max}}$ (μmoles Pi/g. prot./sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unirradiated histidine</td>
<td>$1.27 \times 10^{-4}$</td>
<td>31.9</td>
</tr>
<tr>
<td>Irradiated histidine</td>
<td>$0.81 \times 10^{-4}$</td>
<td>125.0</td>
</tr>
</tbody>
</table>

* The values were calculated from Fig. 1-b.

**Fig. 1.** Effects of irradiated histidine on pH dependency and kinetic property of myosin-ATPase. Myosin solution (0.28 mg protein/ml) was incubated with an equal volume of histidine (5 mM, pH 6.2) in 0.6 M KCl irradiated with 64 kR of γ-rays at 0°C.


**Fig. 2.** Ion-exchange column chromatography of the irradiated histidine solution. Histidine (5 mM, pH 6.2) in 0.6 M KCl was irradiated with 48 kR of X-rays. Chromatographic conditions were given in the text. Solid circles represent ninhydrin-positive substance expressed as histidine equivalent. Open circles represent the activating effect of each fraction expressed as relative ATPase activity. ATPase activity was determined after incubation of myosin (0.14 mg/ml) with each fraction in 0.05 M histidine buffer, pH 6.2 for about 15 hours at 0°C.
peak was negative to diazo reagent, but it exhibited a strong reducing action on Folin’s reagent (Fig. 3-a and -b).

Fig. 4-a shows the ultraviolet absorption spectra of irradiated and unirradiated histidine. In an alkaline medium the spectrum of irradiated histidine shows a rather broad shoulder at about 265 m\(\mu\) and has a higher extinction over the wide range of wave length examined. Fig. 4-b shows spectral change produced by heat treatment in irradiated histidine. The extinction at about 265 m\(\mu\) increases with increasing temperature. This result presumably indicates that the amount of the substance having a peak at about 265 m\(\mu\) in alkaline medium increases on heating.

All results described above suggest that the activating agent separated from irradiated histidine through the column chromatography might be hydroxyhistidine. However, it has been demonstrated by several authors (cf, 20) that by irradiation primidines bases are peroxidized on the C5-C6 bond and converted to hydroxyhydrideroxide in the presence of oxygen, whereas in the absence of oxygen the final main product is a hydroxy compound. Since, in the above experiments, the irradiation of histidine has been
done under aerobic conditions, it seems likely that histidine peroxide is also produced together with hydroxyhistidine. As a first step to ascertain the above inference, the production of sulfhydryl-reactive substance from histidine by irradiation (as a measure for peroxide formation) was examined both under aerobic and anaerobic conditions. As seen from Table 2, a sulfhydryl-reactive substance was produced only when histidine solution was irradiated under aerobic conditions. The formation of a small amount of the substance under anaerobic conditions might be due to the presence of a trace of oxygen. Table 2 also shows that the amount of peroxide by irradiation is much higher under aerobic conditions than under anaerobic.

Next, the amount of peroxide produced by irradiation was compared in histidine solution and distilled water. In both cases the amount increased with increasing dose of X-rays, but no marked difference could be observed in the amount of peroxide formed (Fig. 5-a), although a marked difference was observed in their reactivities with sulfhydryl group of cysteine (Fig. 5-b). In a separate experiment using the column chromatography, it was also found that the peroxide was present in a first small peak containing the activating agent(s).

To distinguish between peroxide formed by irradiation of histidine and of distilled water, the amount of peroxide formed and the reactivity to sulfhydryl group were determined after allowing them to stand at various temperatures. As seen from Table 3, at 0°C-incubation the molar ratio of the decreased sulfhydryl group of cysteine to peroxide formed in the irradiated histidine solution was about three time higher than that of irradiated distilled water. The ratio was diminished by incubation at 37°C in the case of irradiated histidine, while the ratio in irradiated distilled water was not affected much by incubation at 37°C or 100°C. A solution of hydrogen peroxide exhibited heat stability similar to that of irradiated water. These results suggest that peroxide formed from histidine by irradiation may not be hydrogen peroxide itself, but rather a peroxide of histidine.

Table 2. Oxygen effect on the formation of sulfhydryl-reactive substance from irradiated histidine

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cysteine-SH unreacted**</th>
<th>Per cent</th>
<th>Peroxide formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (10^-4M)</td>
<td></td>
<td>(10^-4M)</td>
</tr>
<tr>
<td>Irradiation*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>104.0</td>
<td>61.6</td>
<td>46.0</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>158.4</td>
<td>94.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Unirradiation</td>
<td>168.8</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* Histidine (5 mM, pH 6.2) in 0.6 M KCl solution was irradiated with 48 kR of X-rays under aerobic or anaerobic condition.

** One ml of irradiated solution was incubated with 1 ml of 170 μM cysteine in 15 mM histidine buffer, pH 6.2, in a final volume of 3.0 ml under aerobic condition at 0°C for 3 hours and then the amount of sulfhydryl groups unreacted was determined by DTNB-method.
Fig. 5. Formation of peroxides in the histidine solution and distilled water by X-irradiation and their reaction with cysteine. Histidine (5 mM, pH 6.2) in 0.6 M KCl solution or deionized glass-distilled water was irradiated with X-rays. Determination of peroxide as in “Methods”. (a): The amount of peroxide formed from histidine (●) or from distilled water (○) as a function of X-ray dose. (b): Decrease of sulphydryl group of cysteine after incubation with irradiated histidine solution (●) or with irradiated distilled water (○) at 0°C for 3 hours.

Table 3. Thermo-stability of radiation-induced peroxide from histidine or distilled water and their reactivities with cysteine

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Irradiation doses (kR)</th>
<th>Temperature of treatment (°C)</th>
<th>Peroxide formed (10^{-4}M)</th>
<th>Cysteine-SH unreacted (10^{-4}M)</th>
<th>Cysteine-SH decreased (10^{-4}M)</th>
<th>Decreased SH Peroxide</th>
</tr>
</thead>
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<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>165.6</td>
<td>--</td>
<td>--</td>
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<tr>
<td>Histidine</td>
<td>48</td>
<td>0</td>
<td>177</td>
<td>72.4</td>
<td>93.2</td>
<td>0.53</td>
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<tr>
<td></td>
<td>48</td>
<td>37</td>
<td>178</td>
<td>115.2</td>
<td>50.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>164.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>157</td>
<td>138.3</td>
<td>25.6</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>37</td>
<td>151</td>
<td>135.2</td>
<td>29.2</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>100</td>
<td>130</td>
<td>128.8</td>
<td>35.6</td>
<td>0.27</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0</td>
<td>500</td>
<td>102.0</td>
<td>62.0</td>
<td>105.6</td>
<td>58.4</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>490</td>
<td>105.6</td>
<td>58.4</td>
<td>105.6</td>
<td>58.4</td>
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<tr>
<td></td>
<td>100</td>
<td>464</td>
<td>105.6</td>
<td>58.4</td>
<td>105.6</td>
<td>58.4</td>
</tr>
</tbody>
</table>

* Histidine (5 mM, pH 6.2) in 0.6 M KCl or distilled water was irradiated with 48 kR of X-rays and allowed to stand for 1 hour at 0°C, 37°C and 100°C, respectively.
** The amount of peroxide was estimated immediately after standing.
*** After incubation of 1 ml of resulting solution with 1 ml of 170 μM cysteine at 0°C for 3 hours in 15 mM histidine buffer, pH 6.2, in a final volume of 3.0 ml, the amount of sulphydryl groups unreacted was determined.

Fig. 6 a shows the changes in the amount of peroxide in the irradiated histidine solution, occurring at 0°C after the addition of a low concentration of cysteine. The amount decreased rapidly upon the addition, but it recovered slowly on further incubation. Corresponding to such a change in the amount of peroxide, most of the sulphydryl group of cysteine added disappeared rapidly in the first phase and later only very slowly. The second addition of cysteine to incubation mixture containing the recovered peroxide, (1 hour after the first addition), caused again a rapid drop in the amounts of peroxide formed, and an appreciable amount of cysteine added also disappeared rapidly. In this case, however, the recovery of peroxide diminished to about 20%
of that observed after the first addition. In the absence of cysteine, the amount of peroxide in the irradiated histidine solution decreases very slowly. When cysteine was added to irradiated distilled water (Fig. 6-b) or to a solution of hydrogen peroxide (Fig. 6-c), an initial decrease was also observed, but the recovery of peroxide could not be seen during incubation. From these results it is conceivable that on irradiation a peroxide of histidine which reacts strongly with the sulfhydryl groups of cysteine is produced together with an intermediate which also reacts with cysteine and thus results in the formation of peroxide.

**Comparison of the activating effect of irradiated histidine and that of sulfhydryl reagents on myosin-ATPase:** Since the study of Kielley and Bradley (2), it is well known that the effect of PCMB on the activity of myosin-ATPase is biphasic. In the range of lower concentration of PCMB, the activity increases with increasing concentration and reaches a maximum when 50% of sulfhydryl groups in myosin, 5 moles of sulfhydryl groups per 10^8 g protein, have been titrated. Further addition of PCMB causes inactivation of the enzyme and leads to complete inhibition, when all sulfhydryl groups are titrated. Fig. 7 represents ATPase activity of PCMB titrated myosin, after being incubated with irradiated histidine at 0°C for 6 hours. It can be seen that the activity curve vs PCMB concentration is no longer biphasic. Activation of myosin-ATPase due to irradiated histidine becomes less with increase

![Fig. 6](image_url)

**Fig. 6.** The change of peroxide and cysteine-SH during incubation of irradiated histidine, irradiated distilled water or hydrogen peroxide with cysteine. One ml of histidine (5 mM, pH 6.2) or deionized glass-distilled water irradiated with 64 kR of γ-rays was incubated with 1 ml of 70 μM cysteine in 15 mM histidine buffer, pH 6.2, in a final volume of 3.0 ml at 0°C. At arrow in (a), 2 mM cysteine was added. (a): Irradiated histidine. (b): Irradiated distilled water. (c): Hydrogen peroxide solution.

○: The amount of peroxide in the absence of cysteine.

●: The amount of peroxide in the presence of cysteine.

△: The amount of sulfhydryl group in the non irradiated histidine or distilled water.

▲: The amount of sulfhydryl group in the irradiated histidine, irradiated distilled water or hydrogen peroxide.
ACTIVATION OF MYOSIN-ATPASE BY IRRADIATED HISTIDINE

Fig. 7. Effect of irradiated histidine on the activity of myosin-ATPase treated with PCMB. Histidine (3 mM, pH 6.2) in 0.6 M KCl irradiated with 72 kR of X-rays was incubated with equal volume of PCMB-treated myosin at 0°C for 6 hours. Myosin (0.2 mg protein/ml) was incubated with PCMB at 0°C for 20 min. ATPase activity was determined at pH 7.6.

○: Myosin-ATPase activity incubated with non-irradiated histidine after being treated with PCMB.

Δ: Spectrophotometric titration of sulfhydryl groups of myosin with PCMB after Boyer's method, expressed as increase of optical density at 250 mμ.

Fig. 8. The ATPase activity and the amount of sulfhydryl groups in myosin as a function of the concentration of irradiated histidine. Histidine (3 mM, pH 6.2) in 0.6 M KCl was irradiated with 96 kR of X-rays and added to the myosin solution. The final concentration of the enzyme was 0.12 mg protein per ml. After incubation at 0°C for 20 hours, the ATPase activity was assayed. To determine sulfhydryl groups, Tris-HCl buffer, pH 8.0 and 0.05 ml of DTNB were added to the incubation mixture. At the end of 1 hour at 0°C, sulfhydryl groups were determined. The concentration of irradiated histidine in abscissa was expressed as histidine equivalent amount.

○: ATPase activity.

●: The amount of sulfhydryl groups.

of PCMB. The activity in the higher concentration of PCMB is somewhat lower than that of myosin treated only with PCMB. Similar result was also obtained when myosin treated with irradiated histidine was titrated with PCMB.

Fig. 8 shows that when myosin is incubated with varying amounts of histidine previously irradiated, the activity exhibits a biphasic curve and sulfhydryl groups of myosin decrease with increase in the concentration of irradiated histidine. These
effect are very similar to those of PCMB on myosin. However, 2.5 moles of sulfhydryl groups per $10^6$ g protein, instead of 5 moles, disappeared when the activity reached a maximum and the maximum activity was considerably higher than that of myosin activated by PCMB. These observations seem to suggest that myosin-ATPase activated by irradiated histidine is very similar to NEM-activated ATPase.

As a first step to confirm this point, myosin treated with NEM was incubated with irradiated histidine and ATPase activity was measured in the presence of Ca$^{2+}$ or EDTA as a function of incubation time. As shown in Fig. 9, the activity of NEM-activated ATPase in the presence of Ca$^{2+}$ is no longer enhanced, but decreases during incubation with irradiated histidine (Curve 1). When myosin-ATPase is activated in the presence of EDTA, instead of Ca$^{2+}$, addition of irradiated histidine only causes inactivation of the enzyme (Curve 4). It must be noticed, however, that although NEM almost completely inhibits EDTA-activated ATPase (Curve 3, see Ref. 8), the inhibitory effect of irradiated histidine on EDTA-activated ATPase is not so complete (Curve 4).

To examine the possibility that the activation of ATPase by irradiated histidine may be due to binding of the activating agent with sulfhydryl groups of myosin, irradiated histidine$^{14}$C (after standing at 0°C or 37°C) was incubated with myosin. After incubation, sulfhydryl groups of myosin were measured together with the radioactivity of material insoluble in trichloroacetic acid. From the radioactivity values the binding

![Fig. 9. Effect of irradiated histidine on NEM-activated myosin-ATPase. Histidine (5 mM, pH 6.2) in 0.6 M KCl irradiated with 72 kR of X-rays. NEM-treated myosin: Myosin (9.2 mg protein/ml) was incubated at 0°C for 30 min. with NEM (0.5 mmole/ml) in 0.6 M KCl containing 25 mM Tris-histidine buffer, pH 7.0, in a final volume of 5.0 ml; to remove excess NEM, 5 mM β-mercaptoethanol was added. The mixture was diluted with 10 volumes of distilled water and centrifuged at 10,000 g for 15 min. The resulting precipitate was washed twice with 10 volumes of distilled water. The washed precipitate was dissolved in 20 ml of 0.6 M KCl. An aliquot of the solution was incubated with equal volume of irradiated histidine solution at 0°C. Curve 1: ATPase activity of NEM-treated myosin in the presence of Ca$^{2+}$. Curve 2: ATPase activity of non-treated myosin in the presence of Ca$^{2+}$. Curve 3: ATPase activity of NEM-treated myosin in the presence of EDTA. Curve 4: ATPase activity of non-treated myosin in the presence of EDTA.]
ACTIVATION OF MYOSIN-ATPASE BY IRRADIATED HISTIDINE

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capacity was calculated as histidine equivalent per 10^6 g protein. As shown in Fig. 10, the value of decreased sulphydryl groups per 10^6 g protein is much higher than the binding amount of irradiated histidine. Moreover, reactivity of irradiated histidine with sulphydryl groups is more or less stable at 0°C, whereas standing at 37°C slightly facilitates the binding capacity of irradiated histidine. Although there remains the possibility that irradiated histidine bound to myosin is removed by acid treatment, it seems unlikely that binding of irradiated histidine to myosin is not directly related to the disappearance of sulphydryl groups.

DISCUSSION

In the present study, the activation of myosin-ATPase by irradiated histidine has been examined. All results obtained are consistent with the idea that histidine is converted by irradiation into some type of activating agent for myosin-ATPase and the activation is produced by the interaction of the activating agent with sulphydryl groups of myosin.

It has also been shown that the activating agent in question would not be hydrogen peroxide, but a radiation-induced peroxide of histidine. This is based on the following observations: (a) Peroxide formed from irradiated histidine is different from that formed from irradiated distilled water and hydrogen peroxide in the reactivity to sulphydryl groups and in heat stability. (b) An activating agent which is also reactive to sulphydryl groups can be isolated from irradiated histidine solution through a column chromatography on ion-exchange resin. Judging from its strong reactivity to Folin's reagent and spectral characteristics, the active substance was first supposed to be hydroxyhistidine. However, the hydroxyhistidine might be regarded as a relatively stable end product which was derived from

Fig. 10. The binding capacity of irradiated histidine on myosin and the loss of sulphydryl groups of myosin by irradiated histidine. Histidine 2-14C in 0.6 M KCl (0.2 μCi/ml, pH 6.2) was irradiated with 72 kR of X-rays and allowed to stand for 1 hour at 0°C and 37°C, respectively. Myosin (3.9 mg protein/ml) was incubated at 0°C for 20 hours with irradiated histidine of varied concentrations. An aliquot of the reaction mixture was subjected to determination of sulphydryl groups. To the remainder was added 5% trichloroacetic acid (TCA) and resulting precipitate was washed three times with 1% TCA. The radioactivity of the washed precipitate was determined with a gas flow counter.

▲, ○: The amount of sulphydryl groups.
△, □: The amount of binding as histidine equivalent.
a more labile radiation product, presumably a peroxide. The fact that reactivity with Folin's reagent and absorption at 265 m\(\mu\) of the irradiated histidine increase on heating, accompanied by a decrease in the activating ability on myosin-ATPase, strongly supports this view.

Scholes et al\(^{(21)}\), and Latarjet group\(^{(22-24)}\) has reported that irradiation of pyrimidines in aqueous solution under aerobic conditions causes hydroxyhydroperoxidation at \(\text{C}_4-\text{C}_8\) bond of pyrimidine ring. Since both irradiated histidine and imidazole are effective in activating myosin-ATPase\(^{(15)}\), it seems reasonable to assume that by irradiation of these compounds hydroxyhydroperoxidation takes place at the \(\text{C}_4-\text{C}_8\) bond of imidazole ring and leads to the formation of histidine (or imidazole) hydroxyhydroperoxide. As shown in Fig. 6-a, peroxide in the irradiated histidine solution is rapidly decomposed by the addition of a definite amount of cysteine and then recovery occurs on further incubation. Hence, there is the possibility that either the hydroxyhydrohistidyl radical or the hydroxyhydroperoxyhistidyl radical or both might be produced prior to the formation of hydroxyhydroperoxide\(^{(20)}\).

In the comparative studies, it has been found that the effects of irradiated histidine on myosin-ATPase are very similar to those of sulfhydryl reagents: (a) Irradiated histidine shows an additive effect with PCMB on the ATPase activity; (b) titration of myosin with irradiated histidine shows a biphasic curve, accompanied by a decrease in the amount of sulfhydryl groups of myosin; (c) \(\text{Ca}^{2+}\)-ATPase fully activated by NEM is inhibited by further addition of irradiated histidine; (d) As was found in the case of NEM\(^{(8)}\), irradiated histidine inhibits the EDTA-activated ATPase.

From the studies of the effect of NEM on EDTA-activated ATPase, Sekine\(^{(25)}\) has recently shown that a sulfhydryl group in the active site of myosin molecule, an allosteric site, is concerned with the conformation of the molecule, whereas another sulfhydryl group in the catalytic site is essential for the catalytic action of the enzyme. From this and from observations described above, it may be conclude that an organic peroxide is formed from histidine on irradiation, and that this attacks the sulfhydryl group of the allosteric site of myosin thus causing a new conformation which would facilitate the action of the enzyme, leading to an activation of myosin-ATPase.

However, it appears from Fig. 10 that disappearance of the sulfhydryl groups of myosin caused by irradiated histidine may not be ascribed to binding, but to oxidation of sulfhydryl groups by peroxide. On the other hand, it is well known that PCMB or NEM is a covalent bond-forming sulfhydryl reagent. Thus it is likely that the conformational change of myosin produced by these reagents may differ from that induced by irradiated histidine.

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ACTIVATION OF MYOSIN-ATPASE BY IRRADIATED HISTIDINE

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