FURTHER STUDIES ON THE ISOZYMIC FORMS OF SOLUBLE RAT LIVER GUANINE DEAMINASE

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Introduction

A foregoing communication from this laboratory dealt with the occurrence of soluble rat liver guanine deaminase in two isozymic forms, one of which was an allosteric protein (Sree Kumar and Krishnan, 1970). The effects of sulfhydryl group containing —— and blocking —— agents and of protein denaturing agents have since been investigated; these studies, being reported below, emphasize the fact that while the two enzyme fractions resemble in many properties, quantitative and sometimes qualitative disctinctions exist between the two.

Materials and Methods

Enzyme preparation

Fraction A and fraction B enzyme were prepared as reported earlier (Sree Kumar and Krishnan, 1970).

Enzyme assay

The enzyme assay was as reported (Sree Kumar and Krishnan, 1970), with the difference that on termination of incubation the mixture was acidified with perchloric acid prior to absorption measurement. The unit of enzyme activity and the

specific activity were as already reported.

Agents tested

Sulfhydryl group containing agents, cysteine hydrochloride, glutathione, β-mercaptoethanol and dithiothreitol, were incorporated during the assay, or preincubated with the enzyme at 30°C for 25 minutes, aliquots of which mixture were assayed at intervals. Mercuric chloride was incorporated into the standard assay system. The other sulfhydryl blocking agents, p-chloromercuribenzoate (p-CMB) and N-ethylmaleimide, were preincubated with the enzyme at 30° and at definite intervals aliquots were withdrawn for assay. The effect of N-ethylmaleimide was tested also by its addition during assay. Protein denaturing agents, urea and guanidine hydrochloride, were tested by preincubating the enzymes with the reagents at 30° under specified conditions and withdrawing aliquots for assay at intervals. Solutions of the various reagents were prepared in Tris buffer, pH 8.0, unless otherwise specified.

Results and Discussion

Effect of sulfhydryl group containing agents

Cysteine and β-mercaptoethanol (5×10^{-4} to 1×10^{-1} M), glutathione (1 and 3×10^{-4} M) and dithiothreitol (1 to 5×10^{-3} M) did not influence the enzymic activity.

Incorporated in the assay system in concentrations lower than 3×10^{-5} M, Hg^{2+} exerted negligible or no influence on either enzyme activity. On increasing the concentration, either enzyme was progressively inhibited, till at concentration of 2×10^{-4} M the activity was practically completely inhibited.

Added during assay in 2.5, 5.0 and 7.5×10^{-4} M concentration, p-CMB was without effect on enzyme activity. Marked inhibition

of activity was observed when the enzymes were preincubated with 2.5, 5.0, 7.5 and 10 x 10^{-4} M concentrations of p-CMB. Enzyme 'A' was comparatively less susceptible than 'B' to inhibition by p-CMB. With 7.5 x 10^{-4} M p-CMB, enzyme A was inhibited 50 and enzyme B 80 % when the preincubation period was 10 minutes.

Though the incorporation of N-ethylmaleimide during assay did not affect the enzyme activities, preincubation of enzyme with 2.5, 5 and 10 x 10^{-3} M concentrations of the reagent at pH 8.0 for 15, 30 and 60 minutes elicited significant inhibition. N-ethylmaleimide also had a differential effect on the two isozymes, but the pattern of the response was the reverse of that with p-CMB. Enzyme A was more sensitive than enzyme B to N-ethylmaleimide. Enzyme A was inactivated 50 % and enzyme B only 27 % in 60 minutes at a concentration of 2.5 x 10^{-3} M of N-ethylmaleimide. When the concentration was raised to 5 x 10^{-3} M, for the same period of preincubation, the inactivation of A enzyme was enhanced to 60 % while that of B was essentially unaltered. On increase in the concentration of N-ethylmaleimide to 10 x 10^{-3} M, there was no further increase in the % inactivation of either isozyme.

Tests for reversal by sulfhydryl group containing agents of the inactivation of enzyme by p-CMB and N-ethylmaleimide inhibition by p-CMB

A differential effect was observed in the response by the two isozymes. The inactivation of enzyme A by 2.5 x 10^{-4} M p-CMB could be overcome partially by 5 x 10^{-2} M cysteine and completely by 5 x 10^{-2} M β-mercaptoethanol on 25 minutes exposure of the system to the reducing agents. Complete reversal of the inactivation of enzyme A by 2.5 x 10^{-4} M p-CMB occurred with 2.5 x 10^{-3} M dithiothreitol, a concentration which was one half

Table 1. Reversal by sulfhydryl group containing agents of enzyme inactivation by blocking agents

Additive	Activity, ΔE_{245}	
	Enzyme A	Enzyme B
Nil, control	0.05	0.055
Exposure to 2.5×10^{-4} M p-CMB	0.015	0.01
Inhibited system + 5×10^{-2} M cysteine	0.035	0.02
" + 5×10^{-3} M β-mercaptoethanol	0.035	0.015
" + 5×10^{-2} M β-mercaptoethanol	0.05	0.015
" + 1×10^{-3} M dithiothreitol	0.04	Not tested
" + 2.5×10^{-2} M dithiothreitol	0.045	0.015
Nil, control	0.055	0.055
0.01 M N-ethylmaleimide	0.025	0.04
" + 0.01 M β-mercaptoethanol	0.055	0.045
" + 0.005 M dithiothreitol	0.04	0.05
" + 0.05 M cysteine	0.015	0.045

The enzyme preparation inhibited by p-CMB in 25 minutes was exposed to activator for a period of 25 minutes. The enzyme preparations were treated with N-ethylmaleimide for 60 minutes. This was followed by contact with the sulfhydryl group containing compounds for 20 minutes.

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of that of β-mercaptoethanol to effect total reversal. When the concentration of p-CMB in the preincubation mixture was increased to 5×10^{-4} M (15 minutes exposure) and cysteine and mercaptoethanol used in 5×10^{-2} M concentration in reactivation system, it was observed that cysteine was ineffective and β-mercaptoethanol could reactivate only partially. The inactivation caused by

10×10^{-4} M concentration of p-CMB could not be reversed by either cysteine or mercaptoethanol.

Enzyme B could not be reactivated by either cysteine, β-mercaptoethanol or dithiothreitol.

A time-course study was made of the reactivation of enzyme A inactivated by 2.5×10^{-4} M p-CMB. Using 5×10^{-2} M cysteine, it was observed that the reactivated activity reached a maximum in 5 minutes, thereafter falling off gradually; at the end of 35 minutes the activity dropped to the same level as without activator. With 5×10^{-2} M β-mercaptoethanol as the reactivator, the activity at the end of 5 minutes was 40 % higher than the untreated enzyme. With further incubation, there was a drop in activity and at the end of 35 minutes the activity was the same as that of the untreated but fully active enzyme.

inhibition by N-ethylmaleimide

The preincubation of enzyme was for 60 minutes with 1×10^{-2} M N-ethylmaleimide, when enzyme A and B were inhibited 55 and 27 % respectively. Subsequently it was incubated for 20 minutes with the reactivator. The effect of activator on the inhibited system was tested also by adding it directly to assay system.

Dithiothreitol (1×10^{-3} M) brought about immediate and almost complete reversal of the inactivation of enzyme A. Cysteine and β-mercaptoethanol did not show any instantaneous reversal when tested at 5×10^{-2} M and 1×10^{-2} M respectively. In the preincubated system, β-mercaptoethanol also completely reversed the inhibition. In the presence of cysteine, however, there was further decrease in the activity of the enzyme.

The inhibition of enzyme B by N-ethylmaleimide, which was of a lower order (27-30 %), could not be reversed by any of the

reducing agents, resembling in this respect the irreversibility of inactivation by p-CMB.

Thus, studies on the reactivation of sulfhydryl group — blocked enzyme fractions revealed a distinction in response by the two enzyme fractions; the inhibition of A was reversible, but that of B was irreversible.

Effect of protein denaturing agents

effect of urea

Enzyme A showed an instantaneous drop of 40 % activity with 6 and 8 M urea, dissolved in 0.1 M phosphate buffer, pH 7; there was no further decrease on 30 minutes exposure. Lower concentrations did not result in any immediate inactivation, but storage for 30 minutes with 1 to 4 M urea resulted in 22 % decrease in activity. An exposure period of 2 hours with 8 M urea led to 80 % inhibition of activity, while 4 hours contact at this concentration completely abolished the activity. On 4 hours contact with 1 and 2 M urea 44 % inactivation was observed, while an identical contact period with 4 and 6 M resulted in more than 50 % inactivation.

There was instantaneous drop of 40 % activity also when enzyme B was exposed to 8 M urea in phosphate buffer, pH 7; no further inactivation occurred on 30 minutes contact, but there was complete inactivation in 4 hours. Results of exposure to lower concentrations of urea revealed that the response by enzyme B contrasted with that by enzyme A; enzyme B was more resistant to urea than A. The lower concentrations of urea did not effect any change in the activity on 30 minutes contact. Four hours of contact with 1 and 2 M urea resulted only in a negligible decrease in activity (11 %); the use of 4 and 6 M led to 33 and 52 % inactivation.

The degree of inactivation of enzyme A by urea was dependent

on the pH of medium; with 60 or 90 minutes period of exposure, enzyme was more resistant to inactivation at pH 7.0 than at acidic and alkaline pH.

effect of guanidine hydrochloride

The pattern of relative sensitivity obtained with urea was reversed when guanidine was used as the denaturant. At the highest concentration of denaturant (4 M in Tris buffer, pH 8.0), added during assay, there was 20 % inactivation of enzyme A, contrasting with 80 % inactivation of enzyme B. Enzyme B lost almost complete activity on 30 minutes contact with 2 M guanidine at pH 8.0, whereas A enzyme was only 63 % inactivated.

Reversal of the inactivation of enzyme by urea and guanidine hydrochloride

The inactivation of the enzymes by guanidine hydrochloride could be reversed by one or more treatments, though to different extents.

Ten-fold dilution of the treated enzymes with water or treatment of the diluted denatured enzyme with 1×10^{-6} M guanine or 1×10^{-3} M EDTA or dialysis of the denatured enzyme preparations did not serve to restore the activity of the enzyme. On incorporating reducing agents in the diluted system, the isozymes exhibited qualitative similarity in their reversal, but there were quantitative differences. Complete activity of enzyme B was recovered with β-mercaptoethanol; the inhibition of enzyme A was not only reversed totally, but there was 20 % stimulation of the enzymic activity. While dithiothreitol restored completely the activity of B enzyme, it could restore only 80 % of the activity of A enzyme. Cysteine failed to reverse the denaturation of either enzyme.

The incorporation of 0.001 M Mg^{2+} resulted in 70 % reacti-

Table 2. Reversal by reducing agents and effectors of enzyme inactivation by guanidine

Additive	Activity, ΔE_{245}	
	Enzyme A	Enzyme B
Nil, control	0.05	0.05
Enzyme + guanidine 0 minutes	0.05	0.05
" 30 minutes	0.02	0.005
Treated enzyme diluted	0.02	0.02
Diluted enzyme + 0.01 M cysteine	0.025	0.025
" + 0.001 M β-mercaptoethanol	0.06	0.05
" + 0.005 M dithiothreitol	0.04	0.045
" + 0.001 M Mg^{2+}	0.035	0.035
" + 0.0001 M GTP	0.045	0.02

The enzyme preparations were incubated for 30 minutes with 2 M guanidine hydrochloride, at pH 8.0. Enzyme A was diluted 20-fold and enzyme B 10-fold and assayed as such or with added supplements, after storage for 30 minutes.

vation of both isozymes. On incorporation of 0.0001 M GTP in the medium only A enzyme could be reactivated.

None of the above treatments served to reactivate enzyme A and B denatured by urea.

Thus reactivation of the enzymes denatured by guanidine furnished not only quantitative but also qualitative distinction between the two enzyme fractions.

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Bibliography

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