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INHIBITION OF CHOLINE ACETYLTRANSFERASE FROM BOVINE CAUDATE NUCLEUS BY SULFHYDRYL REAGENTS AND REACTIVATION OF THE INHIBITED ENZYME

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Abstract—Partly purified choline acetyltransferase from bovine caudate nucleus was inhibited by a number of alkylating, oxidizing and mercaptide-forming sulfhydryl reagents. Substrates and products of the choline acetyltransferase reaction had no or, in the case of acetyl-coenzyme A, only a slight protective effect against the inhibition given by 5,5′-dithiobis(2-nitrobenzoate). Reactivation of the enzyme inhibited by the latter compound or by *p*-mercuribenzoate was achieved with thioglycolate; more efficiently if the inhibition and reactivation was performed at 0° than at higher temperatures.

CHOLINE acetyltransferase (acetyl-CoA: choline *O*-acetyl-transferase EC 2.3.1.6) has been shown to be inactivated by certain sulfhydryl reagents. Thus Berman Reisberg[1, 2] demonstrated that the enzyme from squid head ganglia was inactivated by iodoacetate, PMB,† *o*-iodosobenzoate and copper sulfate, and Potter *et al.*[3] recently reported that the enzyme from rat brain was inhibited by copper sulfate and DTNB,† although the effect of the latter reagent was not quantitatively evaluated. Inhibition of choline acetyltransferase from the mollusc *Aplysia* by iodoacetate, *N*-ethylmaleimide and PMB was also recently reported.[4] On the other hand the enzyme from heads of *Musca domestica* is reported to be only slightly sensitive to iodoacetate.[5]

The present paper extends these earlier scattered findings to a more complete investigation on the effects of different sulfhydryl reagents on the partly purified enzyme from bovine caudate nucleus. The inhibitors were chosen to represent the three well-known classes of sulfhydryl reagents,[6] i.e. oxidizing, mercaptide-forming, and alkylating compounds.

An important confirmation of the presence of a sulfhydryl group in an enzyme is the reactivation of the inhibited enzyme by a thiol. Berman Reisberg[2] attempted reactivation of the *o*-iodosobenzoate-inhibited enzyme from squid head ganglia with some success, although no quantitative data were given. In the present communication the conditions necessary for the successful reactivation of PMB- and DTNB-inactivated enzyme are described. Preliminary results of this work have been reported.[7]

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† Abbreviations: PMB, sodium *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

EXPERIMENTAL

Chemicals

The following compounds were obtained from the suppliers indicated: *N*-ethylmaleimide (Schuckardt, recrystallized twice from ethanol); *N*-methyl-, *N*-isopropyl-, *N*-butyl, and *N*-phenylmaleimide, sodium *o*-iodosobenzoate and formamidine disulfide dihydrochloride (Nutritional Biochemicals Corporation); *N*-3,5-dinitrophenylmaleimide (a gift from U Östner; Wenner-Gren Institute, Stockholm, cf. ref. 8); *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide, 2,2′-dithiodipyridine, 4,4′-dithiodipyridine, and DTNB (Aldrich); cystamine dihydrochloride (Calbiochem); PMB, D-pantethine, coenzyme A, iodoacetic acid, and iodoacetamide (Sigma); 4-thiouridine disulfide (Cyclo Chem. Co.). Diphenylcyanoarsine was obtained from the organic chemistry department of this institute. Sodium tetrathionate was synthetized according to Gilman *et al.*[9] Coenzyme A disulfide was prepared by air oxidation of a 20 mM CoA solution in water, adjusted to pH 8·5 with sodium hydroxide.

Labelled acetyl-CoA was prepared as follows: 40 μmoles of CoA was dissolved in 10 ml of 0·1 M $NaHCO_3$ and treated at 0° with 100 μmoles of labelled acetic anhydride (specific activity 1 mc/m-mole, prepared by diluting 1-^{14}C-acetic anhydride (30 mc/m-mole) obtained from the Radiochemical Centre, Amersham, with unlabelled acetic anhydride). When the sulfhydryl reaction (with DTNB) was negative the reaction mixture was acidified to pH 3 by the addition of about 10 g Dowex 50 W-X8 (100–200 mesh, hydrogen form). The suspension was poured into a glass column and the resin was then washed with 20 ml of water. The effluent was concentrated on a rotary evaporator. The residue was dissolved in water and purified by ion exchange chromatography on DEAE-Sephadex A-25 as described for the mixed disulfide of CoA and glutathione.[10] The effluent was monitored at 254 nm, and the major u.v.-absorbing component, which contained most of the radioactivity was collected. When this fraction was analyzed for acetyl-CoA by an enzymatic method,[11] the value obtained accounted for all the u.v.-absorbing material present. It should be noted that acetyl-CoA purified by chromatography on DEAE-Sephadex was found to give 1·7-fold higher activity values in the choline acetyltransferase assay in comparison with the crude acetylated product. All experiments reported in this paper were carried out with the purified acetyl-CoA.

Determination of choline acetyltransferase activity

The activity of choline acetyltransferase was determined in a system similar to that described by Schrier and Shuster.[12] The assay is based on the determination of (1-^{14}C) acetylcholine formed from (1-^{14}C) acetyl-CoA and choline. The product is separated from the labelled substrate by ion-exchange chromatography. The assay system contained 5 mM choline chloride, 0·16 mM (1-^{14}C) acetyl CoA (0·5 mc/m-mole), 0·25 mM eserine salicylate, 0·25% *n*-butanol, 0·3 M NaCl and 0·05 M potassium phosphate pH 7 (giving a final pH of 6·8). The components were mixed in a semimicro centrifuge tube, and the volume was adjusted to 0·2 ml with water. The reaction was started by addition of enzyme (5–40 μl) or substrates, and was carried out at 30° for 20 min. Zero order kinetics were obtained for this period of time, provided that less than 15 per cent of the acetyl-CoA was utilized. The incubation was terminated by a rapid transfer with a Pasteur pipette of the contents in the reaction tube to an ion-exchange column, containing 0·5 × 4 cm of Dowex 1-X2 (chloride form, 200–400

mesh) packed in a Pasteur pipette. The tube was immediately washed with 0·5 ml and subsequently with 1 ml of water to elute the acetylcholine from the column. The effluent, 1·7 ml, was collected in a scintillation vial containing 10 ml of tT21 scintillation fluid.[13] The radioactivity of the sample was finally measured in a liquid scintillation counter, and from the value obtained, the amount of acetylcholine formed in the assay system could be calculated.

Purification of choline acetyltransferase

Caudate nuclei were cut out from bovine brains, obtained from a local slaughterhouse, and could be stored at −20° for several months without loss of choline acetyltransferase activity. The enzyme used in the inhibition experiments was purified in the following manner. (All operations were carried out at 4°.)

A homogenate of 30 g of thawed caudate nuclei was prepared in about 100 ml of 1 mM EDTA by homogenisation with a teflon-glass Potter–Elvehjem homogenizer. The specific activity of the homogenate was $8{\cdot}5 \times 10^{-2}$ nmoles/min mg wet wt. The volume was adjusted to 150 ml with 1 mM EDTA, and the pH raised to 8 by slowly adding 1·45 ml of 1 M ammonia with magnetic stirring in an ice-bath. Centrifugation for 20 min at 27,000 *g* gave 104 ml of supernatant, which was adjusted to pH 6 with 0·50 ml of ice-cold 1 M acetic acid. Acidification of the supernatant caused a turbidity to develop, which however had no adverse effects in the following chromatography step. The supernatant was immediately applied to a 2·5 × 12 cm CM-Sephadex C-50 column equilibrated with 10 mM potassium phosphate–1 mM EDTA, pH 6·0. The column was washed with 600 ml of 0·125 M KCl in the same buffer, which eluted red-coloured material from the column. A concave (upwards) gradient from 0·125 to 1·0 M KCl in 10 mM phosphate–1 mM EDTA pH 6·0 was then applied. The effluent, of a total volume of 750 ml, was collected in 5 ml fractions and the enzyme appeared in the effluent at about 0·25 M KCl. The ten fractions of highest specific activity were pooled, distributed in several tubes, and kept in a freezer. The over-all yield of enzyme was 15 per cent and the specific activity was 59 nmoles/min mg protein, corresponding to a purification of about 140-fold from the starting material. (The protein concentration in the purified enzyme was determined from the absorption at 260 and 280 nm.[14])

Inhibition and reactivation experiments

It was decided to preincubate the enzyme with the inhibitor, as the reaction of some inhibitors with the enzyme is time-dependent, and as substrates may protect the enzyme. Furthermore, one of the products (CoA) of the enzymatic reaction is a thiol, which may react with the inhibitor. The inhibition experiments were, unless otherwise indicated, carried out as follows. The enzyme was preincubated with the inhibitor for 20 min in the assay system in the absence of acetyl-CoA and choline. The assay of the enzymatic activity was then started by the addition of these compounds. The determination of activity was carried out as previously described. The sulfhydryl reagents were first tried at 10^{-3}M concentration and if inhibition was obtained, the inhibitor concentration was decreased step-wise by the power of ten, until less than 50 per cent inhibition resulted or down to 10^{-7}M concentration in case of the most potent compounds.

When reactivation of the inhibited enzyme was attempted the reactivator was

added after the 20 min inhibition period and the determination of activity was then started by addition of the substrates.

RESULTS

When the effect of alkylating agents on the choline acetyltransferase was studied (Table 1), inhibition was found with *N*-alkylmaleimides, whereas iodoacetate and iodoacetamide had no effect even at 10^{-3}M. Among the maleimides the methyl, ethyl and *iso*-propyl derivatives were equally inhibitory, whereas *N*-butylmaleimide was slightly more potent. The aromatic maleimides were even more active, the phenyl

TABLE 1. INHIBITION OF CHOLINE ACETYLTRANSFERASE BY ALKYLATING REAGENTS*

Inhibitor	Concentration (M)	Inhibition (%)
Iodoacetate	10^{-3}	0
Iodoacetamide	10^{-3}	0
N-Methylmaleimide	10^{-3}	87
	10^{-4}	35
N-Ethylmaleimide	10^{-3}	92
	10^{-4}	46
N-iso-Propylmaleimide	10^{-3}	92
	10^{-4}	39
N-Butylmaleimide	10^{-3}	98
	10^{-4}	83
	10^{-5}	12
N-Phenylmaleimide	10^{-5}	40
	10^{-6}	6
N-3,5-Dinitrophenylmaleimide	10^{-5}	29
	10^{-6}	0
N-(4-Dimethylamino-3,5-dinitrophenyl)maleimide	10^{-5}	80
	10^{-6}	20

* The enzyme was preincubated with the inhibitor at 30° for 20 min with all components of the assay system (see Methods) except acetyl-CoA and choline present. The remaining enzyme activity was then determined after addition of the substrates. The inhibitor concentrations given are those of the complete system after addition of substrates; the concentrations during the preincubation with inhibitor being higher by a factor of 1·25. The inhibition was always related to the activity of the control (inhibitor omitted) treated in the same way.

and dinitrophenyl derivatives being equally inhibitory and the dimethylamino dinitrophenylmaleimide being the most active agent.

Compounds able to oxidize sulfhydryl groups were next studied (Table 2). Among aliphatic disulfides, glutathione disulfide was not inhibitory, whereas cystamine had a weak inhibitory action. Pantethine and coenzyme A disulfide, which have greater structural similarities with coenzyme A, were about as active as cystamine. Aromatic disulfides (DTNB, 2,2′-dithiodipyridine, 4,4′-dithiodipyridine, and 4-thiouridine disulfide) were stronger inhibitors by several orders of magnitude. Two other strongly oxidizing disulfides (formamidine disulfide and tetrathionate) were also efficient inhibitors, although somewhat less than the aromatic disulfides. Furthermore, it was found that *o*-iodosobenzoate, which is a potent oxidizing agent for sulfhydryl groups,[6]

TABLE 2. INHIBITION OF CHOLINE ACETYLTRANSFERASE BY OXIDIZING REAGENTS*

Inhibitor	Concentration (M)	Inhibition (%)
Glutathione disulfide	10^{-3}	0
Cystamine	10^{-3}	30
Pantethine	10^{-3}	28
Coenzyme A disulfide	10^{-3}	30
DTNB	10^{-6}	85
	10^{-7}	64
2,2′-dithiodipyridine	10^{-6}	89
	10^{-7}	78
4,4′-dithiodipyridine	10^{-6}	91
	10^{-7}	62
4-Thiouridine disulfide	10^{-5}	96
	10^{-6}	86
	10^{-7}	72
Formamidice disulfide	10^{-5}	79
	10^{-6}	35
	10^{-7}	9
Tetrathionate	10^{-5}	68
	10^{-6}	44
	10^{-7}	13
o-Iodosobenzoate	10^{-4}	48

* Conditions as in Table 1.

TABLE 3. INHIBITION OF CHOLINE ACETYLTRANSFERASE BY MERCAPTIDE-FORMING REAGENTS*

Inhibitor	Concentration (M)	Inhibition (%)
PMB	10^{-6}	86
	10^{-7}	0
Diphenylcyanoarsine	10^{-6}	90
	10^{-7}	4
$HgCl_2$	10^{-6}	88
	10^{-7}	0
$CdCl_2$	10^{-3}	100
	10^{-4}	11
$NaAsO_2$	10^{-3}	22
	10^{-4}	0

* Conditions as in Table 1.

was intermediate between the aliphatic and aromatic disulfides in its inhibitory action.

Three mercaptide-forming reagents (*p*-mercuribenzoate, diphenylcyanoarsine, and mercuric chloride) were found to be strong inhibitors (Table 3). Cadmium chloride and sodium arsenite, which have a high affinity for dithiols,[15] but a much lower affinity for monothiols, were fairly weak in their inhibitory action. This indicates that the enzyme does not require a dithiol group for activity.

It should be noted that the purity of the enzyme used in these inhibition experiments may affect the degree of inhibition obtained. Thus the concentration of DTNB necessary to give about 50 per cent inhibition was increased tenfold when an enzyme preparation with a specific activity of about 20 per cent of the standard preparation was used (Table 4).

As it is known that compounds which participate in an enzymatic reaction as substrates or products may protect the enzyme from inhibition, the effect of the reactants of the choline acetyltransferase catalyzed reaction was tested in experiments where the compounds were present during the preincubation with inhibitor (Table 5). It was found that acetyl-CoA gave a slight protection whereas the other substrate and the products had no such effect. It should be noted that incubation with CoA (in the absence of the sulfhydryl reagent) gave an inhibition which has also been observed in other experiments.

TABLE 4. EFFECT OF DTNB ON CHOLINE ACETYLTRANSFERASE OF DIFFERENT PURITIES*

Enzyme preparation	Specific activity (nmoles/min mg)	Activity (% of control) in presence of DTNB 10^{-6}M	10^{-7}M
I	59	6	63
II	11	51	110

* Conditions as in Table 1, except that enzyme preparation II was of lower purity.

TABLE 5. EFFECT OF SUBSTRATES AND PRODUCTS OF CHOLINE ACETYLTRANSFERASE ON THE INHIBITION OF THE ENZYME BY DTNB*

Addition	Concentration (M)	Remaining activity (%) No DTNB	With DTNB
—	—	100	14
Acetyl-CoA	$1{\cdot}6 \times 10^{-4}$	102	27
Choline	5×10^{-3}	87	15
Acetylcholine	5×10^{-3}	91	14
CoA	10^{-6}	74	15

* The enzyme was preincubated with 1×10^{-6}M DTNB in the presence of a substrate or product as indicated; other conditions as in Table 1. The determination of remaining enzyme activity was started by the addition of the substrate(s) lacking.

The present results have thus demonstrated that choline acetyltransferase is inhibited by most sulfhydryl reagents, but not by all. In order to obtain further evidence for the presence of sulfhydryl groups in the enzyme necessary for the activity, attempts were made to reactivate the inhibited enzyme by incubation with a thiol compound. This should be possible when an enzyme has been inactivated by oxidizing or mercaptide-forming reagents.[6] A complication arose in this type of experiments. Thiols may be nonenzymatically or enzymatically acetylated with acetyl-CoA to give acetylated products,[16] which may interfere in the assay of choline acetyltransferase. These would be labelled and accounted for as acetylcholine formed in the enzymatic reaction, unless they were retained on the ion-exchange column used in the assay. This complication was avoided by choosing thioglycolate, which contains a negatively charged group, as the reactivating agent. Preliminary experiments showed that if the enzyme was incubated with 10^{-6}M PMB for 20 min at 30° complete inhibition resulted, but no reactivation was obtained if the inhibited enzyme was incubated with 10^{-2}M

thioglycolate for 30 min or 18 hr. However, if the inhibition and reactivation was performed at 0° and the time of incubation with inhibitor decreased (requiring a corresponding increase of inhibitor concentration), extensive reactivation by thioglycolate of the PMB- or DTNB-inhibited enzyme could be achieved (Table 6). The results indicate that the inhibited enzyme is unstable and denatures at the higher temperature.

TABLE 6. REACTIVATION OF INHIBITED CHOLINE ACETYLTRANSFERASE BY THIOGLYCOLATE*

Inhibitor	Concentration (M)	Activity (%) Before	Activity (%) After reactivation
PMB	10^{-5}	8	89
DTNB	10^{-5}	18	92
	10^{-6}	35	90

* The enzyme was first incubated at 0° with the inhibitor for 6 min. Thioglycolate (final concentration 9×10^{-3}M) was then added, where indicated, and the sample incubated for 60 min at 0°. The substrates were then added to the reaction tubes and remaining enzyme activity determined at 30°.

DISCUSSION

The results of the present investigation strongly speak in favour of the presence of a sulfhydryl group in choline acetyltransferase necessary for its activity. This interpretation is not contradicted by the fact that iodoacetate, iodoacetamide and glutathione disulfide did not inhibit even at the fairly high concentration of 10^{-3}M, as these sulfhydryl reagents are sometimes found to react slowly or not at all with enzyme-bound sulfhydryl groups.[17] It should be noted in this connection that Berman Reisberg reported[1] that choline acetyltransferase from squid head ganglion was significantly inhibited by 5×10^{-4}M iodoacetate whereas this compound, as mentioned above, was found by us to be without effect on the enzyme from bovine brain. This discrepancy may indicate a different reactivity of the "active" sulfhydryl groups in the choline acetyltransferase from the two sources. Whether the sulfhydryl group is present at the active center of the enzyme from the bovine brain and participates in the catalytic reaction cannot be decided on the basis of the present findings. The absence of a substantial protection by reactants of the enzymatic reaction against inhibition may indicate that the sulfhydryl group is not at the active site.

It is evident from Tables 1 and 2 that inhibitors, containing aromatic or hydrophobic residues, are especially potent. This may indicate the presence of a hydrophobic site in the enzyme. The recently described reversible choline acetyltransferase inhibitors,[18] which do not react with sulfhydryl groups but contain strongly hydrophobic residues support this interpretation. Charge effects seem on the other hand to be of minor importance for the inhibition of the enzyme by sulfhydryl reagents, as both the anionic tetrathionate and the cationic formamidine disulfide (Table 2) are efficient inhibitors.

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REFERENCES

1. R. BERMAN REISBERG, *Biochim. biophys. Acta* **14**, 442 (1954).
2. R. BERMAN REISBERG, *Yale J. biol. Med.* **29**, 403 (1957).
3. L. T. POTTER, V. A. S. GLOVER and J. K. SAELENS, *J. biol. Chem.* **243**, 3864 (1968).
4. E. GILLER, JR. and J. H. SCHWARTZ, *Science* **161**, 908 (1968).
5. M. BOCCACCI, G. NATALIZI and S. BETTINI, *J. Insect Physiol.* **4**, 20 (1960).
6. F. P. CHINARD and L. HELLERMAN. *Meth. Biochem. Anal.* **1**, 1 (1954).
7. B. MANNERVIK and B. SÖRBO, *Abstr. 6th Meeting FEBS, Madrid*, p. 293 (1969).
8. R. W. BURLEY and T. HAYLETT, *Chem. Ind.* 1285 (1959).
9. A. GILMAN, F. S. PHILIPS, E. S. KOELLE, R. P. ALLEN and E. ST. JOHN, *Am. J. Physiol.* **147**, 115 (1946).
10. B. ERIKSSON, *Acta Chem. scand.* **20**, 1178 (1966).
11. D. J. PEARSON, *Biochem. J.* **95**, 23C (1965).
12. B. K. SCHRIER and L. SHUSTER, *J. Neurochem.* **14**, 977 (1967).
13. M. S. PATTERSON and R. C. GREENE, *Anal. Chem.* **37**, 854 (1965).
14. H. M. KALCKAR, *J. biol. Chem.* **167**, 461 (1947).
15. B. P. GABER and A. L. FLUHARTY, *Quart. Rep. Sulfur Chem.* **3**, 319 (1968).
16. D. MORRIS, *J. Neurochem.* **14**, 19 (1967).
17. E. S. G. BARRON, *Adv. Enzymol.* **11**, 201 (1951).
18. C. J. CAVALLITO, H. S. YUN, J. C. SMITH and F. F. FOLDES, *J. med. Chem.* **12**, 134 (1969).