On the Mechanism of Action of Choline Acetyltransferase

(substrate specificity/D2O isotope effect/photo-oxidation)

STEPHEN F. CURRIER AND HENRY G. MAUTNER

Department of Biochemistry and Pharmacology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111

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ABSTRACT The substrate specificity of choline acetyltransferase (EC 2.3.1.6) isolated from squid ganglia was investigated. The enzyme catalyzed the acetylation of choline and aminocholine but not of homocholine. In D2O solution there was considerable slowing of the transacylation reaction. Photo-oxidation in the presence of methylene blue or rose bengal rapidly inactivated the enzyme, suggesting involvement of a histidine residue in the catalytic site. It seems likely that general-base catalysis by imidazole enhances the ability of enzyme-bound choline (or ammoniumcholine) to react with a thiolester group. Attempts to isolate an acethylthio-enzyme intermediate after incubation with [14C]aetylcoenzyme A were unsuccessful. A possible mechanism for the action of choline acetyltransferase is proposed.

Since acetylcholine plays an essential part in the permeability changes to cations in excitable membranes encountered during the conduction of the nervous impulse, a great deal of research has been centered on the enzymes responsible for the hydrolysis and the synthesis of this ester, acetylcholinesterase and choline acetyltransferase (ChA), respectively. ChA, the enzyme catalyzing the transfer of the acetyl group of acetylcoenzyme A to choline, has, since its discovery by Nachmansohn and Machado (1) in rabbit brain, been found in a wide variety of sources, including squid head ganglia (2, 3), rat brain (4), bovine brain (5), human placenta (6), and microorganisms (7). However, considerable difficulties were encountered in trying to obtain highly active, homogeneous material, ChA being a rather sluggish enzyme which tends to be unstable and which may exist in multiple forms (8–10). However, recently ChA with an activity of 4,000 μmoles/hr per mg was isolated by Husain and Mautner from squid head ganglia (11).

While ChA has been known for a long time, a great many questions regarding its mechanism of action remain unanswered. Evidence has been presented that the ChA catalyzed transacylation from acetylcoenzyme A to choline involves an ordered or a Theorell–Chance mechanism (12–14) with acetylcoenzyme A as the leading substrate. Inhibition studies suggest the involvement of a thiol group in the functions of ChA (4, 15–17). It has also been proposed that imidazole may play a catalytic role in the functions of this enzyme (18, 19). The report that relatively stable acetyl-ChA is formed during the enzymatic transacylation reaction (18) was followed by a recent claim that a [14C]acetyl derivative of ChA, presumed to be a thiolester, may be isolated after treatment of the enzyme with [14C]acetylcoenzyme A or with [14C]-acetylcholine (20). In an attempt to clarify the mechanism involved in the functions of ChA, the interaction of this enzyme with a series of substrates and inhibitors was investigated. Evidence was collected suggesting a general-base mechanism, probably involving an imidazole group in the functions of ChA.

MATERIALS AND METHODS

Substrates and Inhibitors. Samples of choline, cholinethiol, homocholine, homocholinethiol, methoxycholine, methylthiocholine, choline disulfide, and choline diselenide were used if their melting points agreed with literature values; if necessary, the samples were recrystallized (20, 21).

Substrate Assays. ChA of squid head ganglia, partly purified (11) to an activity of 150–200 μmoles/hr per mg was used. Enzyme activity was assayed following the procedure of Schrier and Shuster (23). The assay medium contained 50 mM substrate, 30 nM of acetylcoenzyme A Li salt (P and L Biochemicals) containing [14C]acetylcoenzyme A, specific activity 0.01 mCi/mMole (New England Nuclear Corp.) and prostigmine, bovine serum albumin, ethylenediaminetetracetate (EDTA), dithiothreitol, NaCl, NaPi, and 10 μl of ChA (1.8 mg of protein per ml) in the usual amounts (23) with a final volume of 0.2 ml. The samples were incubated at 32° for 30 min; after chromatographic separation, the labeled acetylcholine formed was determined (23) with a Packard Tri-Carb liquid scintillation counter. All determinations were carried out in triplicate, as were controls lacking ChA.

Inhibition Assays. Samples contained 50 mM choline, 50 mM inhibitor, 100 mM NaCl, as well as the other components noted above. All assays were carried out in triplicate as were blanks lacking ChA or inhibitor.

Deuterium Oxide Assays. All components, except for [14C]-acetylcoenzyme A, were dissolved in 99.8% D2O (Mallinkrodt, NMR grade), yielding a final D2O concentration of 95% in the assay samples. pH and P/D were adjusted with NaPi or NaOH dissolved in water or in D2O. Assays were carried out as above (23).

Photo-Oxidation Studies. To a solution (2.5 ml) containing 0.01% dye (methylene blue, Allied Chemical and Dye Corp., or rose bengal, Fisher Scientific Co.), 0.02 M KPi buffer (pH 7.0), 1 mM EDTA, 0.5 M sucrose, and 2 mg of bovine serum albumin per ml, were added 20 μl of ChA solution (0.45 mg of protein per ml, specific activity 80), which had been dialyzed to remove all dithiothreitol. The solution was then placed in a photo-oxidation chamber and irradiated with a 300-W photo-flood lamp (G.E., BEP), while temperature was maintained at 4.0 ± 0.5°.Air was bubbled slowly through the solution during the irradiation. Controls used diluted, dialyzed enzyme and ChA solution instead of the ChA in the solution. Expected effects were recorded at specified time intervals; the results are given in Table 1.

Abbreviations: ChA, choline acetyltransferase; EDTA, disodium ethylenediaminetetraacetate.
Fig. 1. D₂O isotope effect in enzymic and nonenzymic acetylation of choline chloride. Enzymic reaction in H₂O (---O), enzymic reaction in D₂O (---), and nonenzymic reaction in H₂O and D₂O (--.--).

Without dye and diluted, dialyzed enzyme with dye but without irradiation. ChA activity was assayed as above (23).

Attempt to Isolate [¹⁴C]Acetyl ChA. The procedure of Roskoski (20) was followed, the only modification being the use of ChA derived from squid ganglia (11) with an activity of 3000 mmoles/hr per mg, instead of relatively crude bovine brain enzyme. Our enzyme had no measurable carnitine acetyltransferase activity. Incubation and Sephadex G-50 gel filtration were carried out following the literature procedure (20) with a Buchler Fractomette-200 fraction collector. Fractions of 1 ml were collected and tested for radioactivity and for enzyme activity (23). No radioactivity was found in the enzymically active fractions. The three fractions containing most of the radioactivity were combined and the ultraviolet spectrum of a diluted (1:10) aliquot was determined. The λmax of 257 nm agreed with that of acetylcoenzyme A. This fraction could be used to acetylate choline (23).

RESULTS

Table 1 compares the abilities of choline (pKa, 13.9) homocholine, aminochocline (pKa, 6.9), and cholinethiol (pKa, 7.7) to be acetylated by acetyl CoA at a pH of 7 in the presence and the absence of ChA. Table 2 compares the abilities of homocholine, methoxyhomocholine, methyloxyhomocholine, methlythiocoline, choline disulfide, and choline diselenide to act as inhibitors of this enzyme. Since the various trimethylammonium compounds were available as bromide or iodide salts, the effects of sodium bromide and sodium iodide were studied as well.

The pH (pD) dependence of the acetylation of choline and homocholine, in the presence and absence of ChA, was compared in aqueous and in D₂O solution. These results are summarized in Figs. 1 and 2. Control experiments established that after 30 min when the reactions were quenched for determination of acetyl-product formation, the reactions were still proceeding at their initial rates.

Fig. 3 shows the lack of formation of isolable [¹⁴C]acetyl enzyme after incubation of squid ChA with [¹⁴C]acetyl CoA and Sephadex-gel filtration at a pH of 5.9 following the procedure of Roskoski (20).

Photo-oxidation of ChA in the presence of methylene blue and of rose bengal was studied. These results are shown in Fig. 4, showing the effects of these dyes in the presence and the absence of irradiation.

DISCUSSION

Thioesters, while relatively resistant to hydrolysis, are highly susceptible to aminolysis and thiolysis (24–27). It is not surprising, therefore, that the catalytic action of ChA manifests itself much more efficiently in catalyzing the acetylation of

Table 1. Substrate studies (pH 7.0)*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzymic acetylation</th>
<th>Nonenzymic acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. (nmol)</td>
<td>Enzymic acetylation</td>
</tr>
<tr>
<td>Choline + Cl⁻</td>
<td>50</td>
<td>4.0</td>
</tr>
<tr>
<td>Homocholine + Br⁻</td>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td>Aminochocline + Br⁻</td>
<td>50</td>
<td>2.0</td>
</tr>
<tr>
<td>Cholinethiol + I⁻</td>
<td>50</td>
<td>None detected</td>
</tr>
<tr>
<td>Homocholinethiol + I⁻</td>
<td>50</td>
<td>None detected</td>
</tr>
</tbody>
</table>

* Enzymic acetylation refers to the increase in product formation above the level achieved in the absence of ChA.

Table 2. Inhibitor studies

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percent inhibition of acetylcholine formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline + Cl⁻</td>
<td>0</td>
</tr>
<tr>
<td>Homocholine + Br⁻</td>
<td>0</td>
</tr>
<tr>
<td>Methoxyhomocholine + I⁻</td>
<td>16</td>
</tr>
<tr>
<td>Methyloxyhomocholine + Br⁻</td>
<td>5</td>
</tr>
<tr>
<td>Choline disulfide + Br⁻</td>
<td>55</td>
</tr>
<tr>
<td>Choline diselenide + Br⁻</td>
<td>79</td>
</tr>
<tr>
<td>NaBr</td>
<td>7</td>
</tr>
<tr>
<td>NaI</td>
<td>-3</td>
</tr>
</tbody>
</table>

Substrate and inhibitor concentration 50 mM.
choline than in catalyzing the acetylation of aminocholeic or cholinethiol. Acetylcoenzyme A can undergo rapid thiolysis by cholinethiol without enzymic assistance and undergoes spontaneous aminolyis by aminocholeic relatively efficiently at pH's at which the amino group is not protonated. Thus, it appears that the major role of ChA resides in increasing the reactivity of the hydroxy group of choline with regard to a thiolester group. Similarly, ChA enables aminocholeic to be acetylated at pH's at which the amino group is protonated.

Studies of the ChA-catalysed acetylation of choline and aminocholeic showed a considerable isotope effect in D_2O solution, this being greatest at the pH (pD) at which enzymic activity was optimal. Although such interpretations must be treated with caution (28), such isotope effects may be interpreted as indicating that general-base catalysis with rate-determining proton transfer, rather than nucelophilic catalysis, is taking place (29, 30).

In many hydrolytic enzymes, serine oxygen attack is assisted by general-base removal of the proton of the hydroxy group of serine by imidazole (30, 31). It seemed a reasonable assumption that in ChA imidazole might play a similar role in removal of the proton of the hydroxyl group of choline bound to an anionic site, or in removal of a proton from enzyme-bound ammoniumcholine. It has already been postulated, on the basis of the observation that cupric ion blocks the activity of ChA, that imidazole groups might have a catalytic function in this system (18). A similar claim was made on the basis of the observation that imidazole catalyzes the non-enzymic acylation of choline by acetylcoenzyme A (19). It, therefore, seemed important to establish that imidazole groups are essential to the catalytic activity of ChA.

It has been reported that photo-oxidation of methylene blue and of rose bengal causes inactivation of imidazole; in particular, the latter reagent has been claimed to be relatively specific in inactivating histidine residues (32-34). It can be seen in Fig. 4 that photo-oxidation in the presence of the above dyes does indeed result in rapid inactivation of ChA. While these findings will eventually require careful investigation of the modified protein, the rapid inactivation of the enzyme under these conditions strongly supports the involvement of a histidine residue in the active site.

If one assumes that general-base catalysis by an imidazole group enhances the ability of the hydroxy group of choline to react with a thiolester, the question remains whether this reaction involves the thiolester group of acetylcoenzyme A, or that of an acetythiolester derivative of ChA formed as the result of transacylation subsequent to attachment of acetylcoenzyme A. Evidence has been presented that acetylcoenzyme A is the leading substrate during the enzymic acetylation of choline (13, 14). Several workers have proposed that a thiol group is essential to the catalytic functions of ChA (15-17, 20), since thiol reagents such as iodoacetate, p-chloromercuribenzoate, N-ethylmaleimide, or 5,5'-dithiobis(2-nitrobenzoic acid) inhibit this enzyme. These claims are complicated by considerable species differences in the sensitivity of ChA to such reagents, and by uncertainty whether some of these reagents are truly thiol-specific (34), as well as by difficulties encountered by some (16), but not by other (17) workers in obtaining protection of the enzyme from inhibition by thiol reagents by its substrates or its products. Recently Roskoski claimed isolation of an acetylthioenzyme intermediate after incubation of ChA with [14C]acetylcoenzyme A or with [14C]acetylcholine and passage of the reaction mixture through a Sephadex G-50 column (20).

An attempt was made to isolate an acetythiol ChA intermediate of the highly purified enzyme from squid head ganglia. It can be seen in Fig. 3 that, following Roskoski's procedure, Sephadex-gel filtration resulted in a clean separation of the fractions having enzyme activity from the fractions bearing unchanged [14C]acetylcoenzyme A. It should be emphasized that Roskoski used relatively crude bovine brain enzyme, in contrast to the highly purified squid ganglia enzyme used in our studies.

Considerable specificity must be involved in catalysis by ChA. Thus, as shown in Table 1, homocholine, an analog in which the trimethylammonium and the hydroxy groups are separated by three carbon atoms, is not acetylated in the ChA system, while, in the nonenzymic reaction with acetylcoenzyme A, cholinethiol and homocholinethiol are equally reactive. This finding, coupled with the earlier observation of Hemsworth and Smith (35) that enantiomers of α-methyl and β-methylcholine exhibit some stereospecificity, emphasizes the importance of suitable alignment of the cationic and the
nucleophilic portions of substrate attached to the choline-binding site of ChA.

The data presented in this manuscript seem compatible with a mechanism of action for ChA summarized in Fig. 5. Acetylcoenzyme A is attached to a suitable binding site. Choline is attached to a choline-binding site, and the nucleophilicity of the hydroxy group enhanced by general base catalysis involving an imidazole group. Transacetylation to choline then yields acetylcholine. The question whether an enzymatic thiol group is utilized to form an acetyl-ChA intermediate (20) remains to be clarified.

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