The Reaction of Acetyl-Coenzyme A with Choline Acetyltransferase

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Product-inhibition patterns and coenzyme-binding studies together suggest that acetyl-CoA acts as the leading substrate for choline acetyltransferase (EC 2.3.1.6) in the forward reaction, and CoA likewise in the reverse reaction (Glover & Potter, 1971; Morris *et al.*, 1971; Prince & Hide, 1971; Sastry & Henderson, 1972; White & Cavallito, 1970). CoA and acetyl-CoA also stabilize squid choline acetyltransferase by binding to the free enzyme, whereas neither choline nor acetylcholine is effective (Prince & Hide, 1971). This evidence suggests that CoA and acetyl-CoA control and maintain the active structure of choline acetyltransferase, and that neither choline nor acetylcholine is able to bind to the enzyme until CoA or acetyl-CoA has combined. These conclusions are valid for choline acetyltransferase preparations at high or low ionic strengths, even though ionized salts increase the maximum reaction rates and change the kinetic constants (Prince & Hide, 1971).

We now present evidence that supports a choline acetyltransferase-structure-organizing role for CoA and acetyl-CoA, and that also provides a method, independent of the reaction kinetics, for distinguishing between high-salt and low-salt forms of choline acetyltransferase. Acetyl-CoA and CoA stabilized both squid ganglion and rat brain choline acetyltransferase, and reactivated samples that had been thermally denatured. Not only were the reactivation characteristics of the squid and the rat enzymes different, but the reactivation of the respective high-salt and low-salt activities also differed.

Partially purified squid (*Loligo vulgaris*) choline acetyltransferase was prepared from acetone-dried powders of head ganglia (Prince, 1970; Prince & Hide, 1971). Rat brain choline acetyltransferase was purified 100-fold from homogenates of fore- and mid-brain by ion-exchange chromatography on CM-Sephadex (Glover & Potter, 1971). Assay methods were dictated by the activity and stability of the two enzyme preparations. Initial rates of squid choline acetyltransferase reactions (at 30°C) were measured spectrophotometrically (Prince & Hide, 1971). Rat brain choline acetyltransferase was assayed radiochemically by 1 min incubation (at 37°C) with choline and [$1\text{-}^{14}\text{C}$]acetyl-CoA (specific radioactivity 5.4 or 59 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) as required. Acetylcholine was separated from the residual acetyl-CoA by electrophoresis [4°C; Whatman no. 1 paper; KH_2PO_4

(10 mM), pH 7.0; 75 V/cm; 20 mA; 15 min) and determined on the paper by conventional liquid-scintillation counting procedures. Each enzyme preparation was assayed at low and high ionic strengths in buffers containing KH_2PO_4 (10 mM), EDTA (0.5 mM) and KCl (0 or 150 mM), pH 7.0.

The thermal stabilities of the two choline acetyltransferase samples were different. Incubation at 30°C caused a rapid denaturation of squid choline acetyltransferase, whereas a temperature of 46°C was necessary for comparable rates of denaturation of the rat enzyme. Further, KCl (150 mM) partially stabilized rat choline acetyltransferase, whereas it decreased the stability of samples from squid (Fig. 1). Despite these differences, however, rat choline acetyltransferase, like squid choline acetyltransferase (Prince & Hide, 1971), was stabilized by acetyl-CoA at all ionic strengths, and the stabilization constant (K_{ia}), the Michaelis constants for acetyl-CoA (K_a) and for choline (K_b), as well as the reaction V_{max} value, all increased with ionic strength (Table 1). That acetyl-CoA stabilizes rat as well as squid choline acetyltransferase further supplements the kinetic evidence in favour of the proposed ordered mechanism.

With partially denatured squid choline acetyltransferase, progress curves obtained spectrophotometrically, for reactions with acetyl-CoA (0.15 mM) and choline chloride (10 mM) in the low-ionic-strength assay buffer (30°C), showed marked increases (up to 100%) in the rates of synthesis of acetylcholine over approx. 1 h. The rates of similar reactions in the high-ionic-strength assay buffer, however, decreased, as normally expected from progressive consumption of the coenzyme.

The implication that thermally denatured squid choline acetyltransferase could be reactivated was confirmed as follows. The enzyme was inactivated at high or low ionic strength (at 30°C) for 10 min, then sampled for residual choline acetyltransferase activity under the same conditions, while the remaining solution was incubated (at 30°C) with a saturating concentration of acetyl-CoA or CoA. Incubation at low ionic strength with acetyl-CoA resulted in full recovery of the low-salt activity in 3-5 h, whereas CoA was less effective. Samples that had been more fully denatured also regained activity, but less completely (Fig. 1a). At high ionic strength there was little recovery of the corresponding high-salt activity, and during long incubations with acetyl-CoA activities slowly declined. In contrast, a greater recovery of the

high-salt activity accompanied reactivation at low ionic strength, although the decline in activity during long incubation, a characteristic of the high-salt activity, was retained (Fig. 1a).

Activity lost by storing squid choline acetyltransferase at 4°C, or by deep-freezing, was also recovered by incubation with acetyl-CoA at low ionic strengths. In experiments with such stored samples the enzyme was more active after incubation (1–5 h) with acetyl-CoA than it was before the initial denaturation at 30°C. In this respect therefore aged choline acetyltransferase resembled the denatured enzyme (Prince & Hide, 1971).

Thermally denatured rat brain choline acetyltransferase could also be reactivated by acetyl-CoA, although the conditions differed from those for squid

choline acetyltransferase. No recovery was found at either the temperature required for denaturation (46°C) or at the normal assay temperature (37°C). However, at 1°C the enzyme substantially recovered in 3 h. In contrast with the behaviour of the squid enzyme, but consistent with the high-salt and low-salt stabilities of rat choline acetyltransferase, reactivation was more effective at high than at low ionic strength (Fig. 1b). Surprisingly, a large proportion of this reactivation occurred in the absence of acetyl-CoA at both ionic strengths, and again KCl favoured the recovery. No spontaneous reactivation of squid choline acetyltransferase occurred between 1° and 30°C. Also, in contrast with squid choline acetyltransferase, the recovery of the high-salt activity was not improved by reactivation at low ionic strength (Fig.

Fig. 1. Denaturation and reactivation curves for squid ganglion and rat brain choline acetyltransferase

(a) Squid choline acetyltransferase: initial and residual activities assayed (at 30°C) with choline chloride (10mM) and acetyl-CoA (0.15mM). Buffers for denaturations, reactivations and assays, at low or high ionic strengths, consisted of KH_2PO_4 (10mM), EDTA (0.5mM) and KCl (0 or 150mM), pH 7.0. Acetyl-CoA (0.15mM) or CoA (0.21mM) was used throughout for reactivations. Denaturation and reactivation were both carried out at 30°C. o, Low-salt activity; ●, high-salt activity. Curve A, denaturation, low ionic strength; curve B, denaturation, high ionic strength; curve C, reactivation by acetyl-CoA after 10min denaturation, low ionic strength throughout; curve D, reactivation by acetyl-CoA after 70min denaturation, low ionic strength throughout; curve E, reactivation by acetyl-CoA after 10min denaturation, high ionic strength throughout; curve F, reactivation of high-salt activity by acetyl-CoA (low ionic strength) after 10min denaturation (low ionic strength); curve G, reactivation by CoA after 10min denaturation, low ionic strength throughout. (b) Rat choline acetyltransferase: initial and residual activities assayed (at 37°C) with choline chloride (2mM) and acetyl-CoA (1.7μM, curves A and B; 34μM, curves C–G). Buffers for denaturations, reactivations and assays, at high or low ionic strengths, were as described for experiments with squid choline acetyltransferase; however, denaturation was at 46°C and reactivation at 1°C. Acetyl-CoA (34μM) was used for reactivation. o, Low-salt activity; ●, high-salt activity. Curve A, denaturation, low ionic strength; curve B, denaturation, high ionic strength; curve C, reactivation by acetyl-CoA after 5min denaturation, low ionic strength throughout; curve D, spontaneous reactivation after 5min denaturation, low ionic strength throughout; curve E, spontaneous reactivation after 10min denaturation, high ionic strength throughout; curve F, reactivation by acetyl-CoA after 10min denaturation, high ionic strength throughout; curve G (broken line), spontaneous reactivation (at low ionic strength) of high-salt activity after 5min denaturation (low ionic strength).

Table 1. Maximum activities and Michaelis and stabilization constants for squid ganglion and rat brain choline acetyltransferase at high and low ionic strengths

Enzyme preparation	Substrate	Michaelis constant (K_a or K_b) (μ M)		Stabilization constant (K_{ia}) (μ M)		V_{max} (μ mol of acetylcholine synthesized/min per mg of protein)	
		High-salt	Low-salt	High-salt	Low-salt	High-salt	Low-salt
		(K_a or K_b) (μ M)		(K_{ia}) (μ M)		V_{max} (μ mol of acetylcholine synthesized/min per mg of protein)	
Squid ganglion	Acetyl-CoA	50-70	23-27	28-42	3.0-3.4	1.1-1.4	0.25-0.29
	Choline	2000-3000	700-1000	—	—	—	—
	CoA	—	—	100-150	12-28	—	—
Rat brain	Acetyl-CoA	16.8-17.2	8.5-8.9	7.9-8.1	0.75-0.85	0.09-0.10	0.045-0.055
	Choline	800-900	480-520	—	—	—	—

The temperatures used were: squid choline acetyltransferase, 30°C throughout; rat choline acetyltransferase, kinetics 37°C; stability 46°C. The V_{max} values and the Michaelis constants given are the limiting values. The ranges given represent 95% confidence limits throughout.

1b). Choline and acetylcholine did not stabilize or reactivate either rat or squid choline acetyltransferase.

The recovery of the low-salt activity of the squid enzyme depended on the concentration of acetyl-CoA as well as on the incubation time. A plot of reciprocal reactivation against reciprocal concentration of acetyl-CoA indicated a 'reactivation constant' of approx. 15 μ M. Reciprocal reactivation was defined as $(F-U)/(R-U)$, where F is the choline acetyltransferase activity of a fully reactivated sample, U is the activity of a sample denatured for 10 min (30°C; low ionic strength) and R is the activity after equilibration with acetyl-CoA (6-150 μ M) for 5 h. The reactivation constant calculated in this manner contrasted with the value of 3 μ M (Table 1) obtained for the stabilization constant. Reactivation therefore may not be the result of a shift of equilibrium between active and inactive choline acetyltransferase, caused by binding of acetyl-CoA to the active enzyme. It is likely to be the result of a direct binding to denatured choline acetyltransferase, which is then slowly converted into the active enzyme-coenzyme complex.

Similar conclusions were drawn from the optical-rotation and fluorescence changes accompanying the regain of ordered structure and catalytic activity of several denatured enzymes (Teipel & Koshland, 1971a,b). The denatured proteins rearranged to form a mixture of several conformational isomers in equilibrium during renaturation, either spontaneously or under the influence of a specific ligand. Kinetic or thermodynamic factors, related to the structure of both the native enzyme and the inactive intermediates, determined whether or not the final renatured protein was catalytically active. The difference between the reactivation characteristics, not only for rat and for squid choline acetyltransferase, but also for the respective high-salt and low-salt activities, therefore probably arise from differences in protein structure.

Squid choline acetyltransferase high-salt and low-salt activities showed different reactivation characteristics on incubation of the denatured enzyme preparation with acetyl-CoA at low ionic strength (Fig. 1a). This suggests that more than one discrete form of choline acetyltransferase exists in the preparation. In contrast, spontaneous recoveries of rat brain choline acetyltransferase high-salt and low-salt activities were identical at low ionic strength (Fig. 1b). This is compatible with the presence in our preparation of either a single enzyme activated by salts, or more than one discrete enzyme, with similar reactivation characteristics and salt sensitivities.

Isoelectric focusing has revealed a high degree of heterogeneity of choline acetyltransferase, not only between species, but also within the same species (Malthe-Sørenssen & Fonnum, 1972). These results support our conclusion that the rat and the squid enzymes are appreciably different, and they also

support the interpretation of the results for both the squid and the rat preparations in favour of at least two different enzyme forms. In spite of this, the effect of salts on the maximum catalytic rate and on the initial binding of the coenzymes (Table 1) cannot be explained simply by the existence of several different forms of choline acetyltransferase. However, the differences in salt activation found with choline acetyltransferase of different origins and prepared by different extraction and purification procedures (see, e.g. Table 1; Glover & Potter, 1971; Morris & Tuček, 1966; Potter *et al.*, 1968; Schubert, 1966) could originate from the existence of several forms of choline acetyltransferase with different sensitivities to salts.

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