The Effects of Substrates on the Optical Rotatory Dispersion of Carnitine Acetyltransferase

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1. The optical rotatory dispersion of carnitine acetyltransferase is altered in the presence of L-carnitine or acetyl-L-carnitine. These changes, which include an increase in the reduced mean residue rotation at 233 nm. ($[M']_{233}$), suggest that substrate binding causes the enzyme to unfold. 2. CoA and acetyl-CoA have no immediate effect on $[M']_{233}$ and CoA has no effect on the change in this parameter induced by L-carnitine. 3. The change in $[M']_{233}$ was used as a measure of the degree of saturation of the enzyme with carnitine substrates. Dissociation constants for the enzyme complexes with L-carnitine, D-carnitine and acetyl-L-carnitine were determined in this way. 4. Prolonged incubation of carnitine acetyltransferase in the presence of CoA leads to a small increase in the value of $[M']_{233}$ accompanied by irreversible inhibition of the enzyme. 5. Optical-rotatory-dispersion studies of two specifically inhibited enzyme forms are reported.

The possibility that the formation of an enzyme-substrate complex may be accompanied by a conformational change in the enzyme protein has been made the basis of several theories of enzyme action (Lumry & Eyring, 1954; Karush, 1950; Koshland, 1958). In this work, changes were observed in the O.R.D.* of carnitine acetyltransferase (acetyl-CoA-L-carnitine *O*-acetyltransferase, EC 2.3.1.7) on binding of its substrates L-carnitine and acetyl-L-carnitine. These changes have been used to determine the extent of complex-formation at various concentrations of each substrate. It was hoped that measurement of dissociation constants for the enzyme-substrate complexes in this way might provide evidence in favour of the equilibrium kinetic mechanism with a random order of substrate binding proposed for carnitine acetyltransferase by Chase & Tubbs (1966).

MATERIALS

Crystalline carnitine acetyltransferase and CoA were obtained from Boehringer Corp. (London) Ltd., London W.5. Acetyl-CoA was prepared from CoA by treatment with acetic anhydride (Simon & Shemin, 1953).

D- and L-Carnitine hydrochlorides were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and *O*-acetyl-L-carnitine hydrochloride was prepared from L-carnitine hydrochloride by the method of Fraenkel & Friedman (1957) and recrystallized twice from butanol. Bromoacetyl-L-carnitine and bromoacetyl-CoA were prepared as described by Chase & Tubbs (1969).

The carnitine acetyltransferase-*S*-carboxymethyl-CoA L-carnitine ester complex was prepared from carnitine acetyltransferase, bromoacetyl-CoA and L-carnitine by the method of Chase & Tubbs (1969).

Tris (Trizma Base) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset. Distilled water was passed through a Permutit mark 11 deionizer before use.

METHODS

Assay of enzyme and substrates. Carnitine acetyltransferase activity was measured by adding enzyme samples to a system containing tris-HCl buffer, pH 7.8 (100 mM), acetyl-CoA (0.1 mM) and L-carnitine hydrochloride (1.25 mM) in a final volume of 2.0 ml. The initial rate of decrease of $E_{232}^{1\%}$, due to the deacetylation of CoA, was measured at 30° with a Beckman DK-2A recording spectrophotometer.

Solutions of CoA, acetyl-CoA, L-carnitine and acetyl-L-carnitine were assayed as described by Chase & Tubbs (1966). The concentrations of carnitine acetyltransferase solutions were estimated spectrophotometrically by taking $E_{1\text{cm}}^{1\%}$ to be 8.25 at 280 nm. (Chase & Tubbs, 1969).

Measurement of O.R.D. All measurements were made with a Bellingham and Stanley Polarmatic 62 spectropolarimeter. A 250 W xenon arc lamp was used as the light-source and the apparatus was continuously purged with nitrogen. A 0.098 cm. sample cell was used and the cell compartment was water-jacketed at 30°. Fixed slits were used over the entire wavelength range with the entrance slit set at 1.6 mm. and the exit slit at 1.3 mm. All solutions were filtered through membrane filters (Oxoid Ltd., London E.C.4) before being used. Substrate titration experiments were carried out by adding samples of a concentrated solution of the appropriate substrate up to a

* Abbreviation: O.R.D., optical rotatory dispersion.

total volume not exceeding 12% of the total volume of the enzyme-substrate mixture. The enzyme, carnitine and acetylcarnitine solutions were buffered in 0.2M-sodium phosphate, pH 7.4. The CoA and acetyl-CoA solutions were adjusted to pH 7.4 immediately before use and were kept in ice. Values obtained for the effect of additions of the substrates on the O.R.D. of the buffer solution in the absence of carnitine acetyltransferase were subtracted from the values in the presence of the enzyme and corrections were made for the dilution of the enzyme solution. Corrections for the O.R.D. of the substrates were always very small and never exceed 3% of the value of $[M']_{233}$ observed in the presence of enzyme. The reduced mean residue rotations $[M']$ were calculated from the values of the refractive indices of water as a function of wavelength given by Fasman (1963) and assuming a value of 133 for the mean residue weight of carnitine acetyltransferase calculated from amino acid analysis data (J.F.A. Chase, unpublished work). The Moffitt constants for the enzyme were calculated from O.R.D. data by the graphical method of Urnes & Doty (1961). A value of 216nm. was assumed for the constant λ_0 when measurements in the wavelength range 300–250nm. were used and a value of 212nm. was used for measurements in the wavelength range 350–290nm. (Fasman, 1963).

RESULTS

O.R.D. of carnitine acetyltransferase in the presence and absence of substrates. O.R.D. curves of carnitine acetyltransferase in the presence and absence of L-carnitine are shown in Fig. 1. The presence of L-carnitine caused a decrease in magnitude of both the Cotton-effect trough at 233nm. and the peak at

Fig. 1. O.R.D. of carnitine acetyltransferase determined in the absence (—) and presence (---) of 1.7mM-L-carnitine. Rotation was determined at 30° in a 0.098cm.-light-path cell. Both the enzyme and the L-carnitine samples were buffered in 0.2M-sodium phosphate buffer, pH 7.4.

196nm. The change in $[M']_{233}$ was measured over a range of L-carnitine concentrations and the results are shown as a double-reciprocal plot in Fig. 2.

D-Carnitine, a competitive inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1966), was found to cause changes in the O.R.D. of the enzyme similar to those associated with the L-isomer (Fig. 2).

The presence of CoA or acetyl-CoA, at final concentrations more than 6 times the measured K_m values for these substrates (Chase & Tubbs, 1966), had no effect on the O.R.D. of carnitine acetyltransferase. Additions of L-carnitine to the enzyme in the presence of 250 μ M-CoA (Fig. 3) changed the value of $[M']_{233}$ to a similar extent to that observed in the absence of CoA (Fig. 2). Fig 3 also shows that additions of acetyl-L-carnitine to a solution of carnitine acetyltransferase were accompanied by increases in $[M']_{233}$ that tended asymptotically, at high substrate concentration, to a value similar to that seen with L-carnitine (Table 2).

Dissociation constants calculated from the data of Figs. 2 and 3 are shown in Table 1 and are compared with the corresponding K_m or K_t values determined from kinetic studies. The differences between the K_s values and the K_m (or K_t) values for the various forms of carnitine are no greater than might be expected from the nature of the experimental methods employed. Thus Baker & Mahler (1962) have argued that the errors involved in determining the ratio of two kinetic constants may well approach 30%. The only values in Table 1 which could not be reconciled by such a range of error are the K_s and

Fig. 2. Effect of varying the carnitine concentration on the reduced mean residue rotation of carnitine acetyltransferase plotted as reciprocals of the change in rotation versus the concentration of L-carnitine (●) and D-carnitine (○). The conditions of the experiments were as given in Fig. 1.

Fig. 3. Effect of varying the substrate concentration on the reduced mean residue rotation of carnitine acetyltransferase plotted as reciprocals of the change in rotation versus the concentration of acetyl-L-carnitine (○) and L-carnitine in the presence of 250 μ M-CoA (●). The conditions of the experiments were as given in Fig. 1.

Table 1. Dissociation constants determined from optical-rotation titrations compared with the kinetic constants for the same compounds at pH 7.4 (Chase, 1967a)

Compound	Dissociation Constant	Kinetic constants	
	K_s (μ M)	K_m (μ M)	K_t (μ M)
L-Carnitine	115	158	—
D-Carnitine	106	—	173*
Acetyl-L-carnitine	312	398	—
L-Carnitine in the presence of 250 μ M-CoA	108	—	—

* At pH 7.8 (Chase & Tubbs, 1966).

K_t for D-carnitine; even greater errors may have resulted in this case from the absence of a specific enzymic assay procedure for the standardization of D-carnitine stock solutions.

Table 2 summarizes the values of $[M']_{233}$ found for carnitine acetyltransferase under the various conditions described above. In addition, the Moffitt constants, b_0 , calculated from O.R.D. spectra in the ranges 300–250nm. and 350–290nm. were -88.0 and -92.0 respectively for the native enzyme and -69.7 and -74.0 respectively for the enzyme in the presence of 1.7mM-L-carnitine. The reduced mean residue rotation at 196nm. ($[M']_{196}$) was 12660 ± 720 (six determinations) for the native enzyme; this was decreased to 8036 ± 627 (three determinations) in the presence of 1.7mM-L-carnitine.

O.R.D. studies of two inhibited forms of carnitine acetyltransferase. Bromoacetyl-CoA has been shown to inhibit carnitine acetyltransferase in the presence of L-carnitine with the formation of *S*-carboxymethyl-CoA L-carnitine ester, which is only very slowly released from the enzyme (Chase & Tubbs, 1969). The magnitude of the trough of the 233nm. cotton effect for a sample of enzyme that had been treated in this way was decreased considerably compared with that of the native enzyme and was decreased only slightly by the further addition of L-carnitine (Table 2).

Incubation of carnitine acetyltransferase with the active-site-directed inhibitor bromoacetyl-L-carnitine in the absence of CoA results in irreversible inhibition owing to the formation of an inactive monocarboxymethylated derivative of the enzyme (J. F. A. Chase, P. K. Tubbs & R. N. Perham, unpublished work). The magnitude of the trough of the 233nm. cotton effect for this derivative was a little less than that for the untreated enzyme, but the addition of normally saturating amounts of L-carnitine had no further effect on its O.R.D.

Loss of carnitine acetyltransferase activity on prolonged incubation with CoA. Although CoA caused no immediate change in the O.R.D. of carnitine acetyltransferase, prolonged incubation of the enzyme with 250 μ M-CoA at 30° led to a gradual increase in the value of $[M']_{233}$ that was complete in about 150min. (Table 2). After this incubation no further change in the O.R.D. of the enzyme took place when L-carnitine was added. To determine whether this conformational change of the enzyme protein, brought about by exposure to CoA, was associated with altered catalytic ability, samples were removed at intervals from such an incubation mixture and assayed for carnitine acetyltransferase activity. Fig. 4 shows that there was a progressive and CoA-dependent loss of enzyme activity. Incubation of samples of carnitine acetyltransferase with 250 μ M-acetyl-CoA or 4mM-L-carnitine produced no time-dependent change in the O.R.D. of the enzyme and no significant loss of activity.

The addition of dithiothreitol (final concn. 1mM) to a sample of carnitine acetyltransferase that had been incubated with 250 μ M-CoA for 25min. did not affect the activity of the enzyme, nor did the presence of 1mM-dithiothreitol alter the time-course of the inhibition of carnitine acetyltransferase by CoA.

DISCUSSION

In Fig. 2 it is shown how the change in the value of $[M']_{233}$ for carnitine acetyltransferase at various L-carnitine concentrations may be used to follow the saturation of the enzyme with this substrate, thus enabling the dissociation constant for the

Table 2. *Reduced mean residue rotation at 233nm. ($[M']_{233}$) of carnitine acetyltransferase and its derivatives*

The results are expressed as the means $\pm$ s.e.m. with the numbers of determinations in parentheses.

Enzyme sample	Addition	$[M']_{233}$
Native carnitine acetyltransferase	—	-3114 ± 32 (24)
	1.7 mM-L-Carnitine	-2432 ± 26 (10)
	250 μ M-CoA	-3062 ± 48 (4)
	1.7 mM-D-Carnitine	-2350 ± 50 (3)
	250 μ M-CoA plus 1.7 mM-L-carnitine	-2378 ± 10 (4)
	370 μ M-Acetyl-CoA	-3083 ± 44 (3)
	7 mM-Acetyl-L-carnitine	-2310 ± 42 (3)
	250 μ M-CoA and incubated at 30° for 180 min.	-2847 ± 60 (5)
Monocarboxymethyl-carnitine acetyltransferase	—	-2965 ± 46 (6)
	1.7 mM-L-Carnitine	-2910 ± 30 (5)
Carnitine acetyltransferase-S- carboxymethyl-CoA L-carnitine ester complex	—	-2633 ± 40 (3)
	1.7 mM-L-Carnitine	-2483 ± 12 (3)

Fig. 4. Progressive inhibition of carnitine acetyltransferase by CoA. Incubation mixtures containing potassium phosphate buffer, pH 7.2 (25 mM) and carnitine acetyltransferase (0.45 μ M) with (Δ) or without ($\bullet$) 250 mM-CoA in a final volume of 0.5 ml. were kept at 21°. Samples (50 μ l.) were taken for assay of residual carnitine acetyltransferase activity at the times shown.

enzyme-L-carnitine complex to be calculated. Estimates of dissociation constants have also been made for D-carnitine, for acetyl-L-carnitine and for L-carnitine in the presence of saturating amounts of CoA (Figs. 2 and 3). The reasonable agreement (Table 1) between the dissociation constants for L-carnitine and acetyl-L-carnitine obtained here and the kinetically determined Michaelis constants for these substrates lends support to the kinetic mechanism for carnitine acetyltransferase proposed by Chase & Tubbs (1966). It is a necessary prediction of such a random-equilibrium mechanism that the measured K_m values for each substrate should approximate closely to true dissociation constants. Chase & Tubbs (1966) also found that

the presence of carnitine on its binding site had no effect on the affinity of the enzyme for CoA and vice versa. This is in accordance with the findings (Fig. 3 and Table 1) that the O.R.D. change induced in carnitine acetyltransferase by binding of L-carnitine and the dissociation constant of the enzyme-L-carnitine complex are unaffected by the simultaneous presence of CoA. The validity of using changes in the O.R.D. of the enzyme at various substrate concentrations to determine dissociation constants is supported by the agreement between K_s for the enzyme-D-carnitine complex reported here and the value obtained from kinetic measurement of K_i for D-carnitine (Table 1).

That a conformational change of the enzyme protein might accompany the binding of carnitine to carnitine acetyltransferase has been inferred from earlier kinetic studies (Chase, 1967b). The change in the O.R.D. of carnitine acetyltransferase seen in the presence of carnitine or acetylcarnitine (Figs. 1-3) indicates that the conformation of the enzyme is indeed altered on binding of these substrates. The direction of the change is that expected from unfolding of the protein (Urnes & Doty, 1961).

The O.R.D. of a sample of carnitine acetyltransferase that had been inhibited by treatment with bromoacetyl-CoA plus L-carnitine followed by removal of excess of inhibitor is very similar to that shown by the native enzyme in the presence of saturating amounts of carnitine (Table 2). This form of inhibited enzyme therefore appears to be permanently in the conformation that is normally induced by carnitine binding. The explanation for such inhibition given by Chase & Tubbs (1969),

involving formation at the enzyme surface of very tightly bound *S*-carboxymethyl-CoA L-carnitine ester, is clearly in accordance with such a finding.

No such complete interpretation is possible of the O.R.D. of the other two inhibited enzyme forms that have been studied (Table 2). Thus the O.R.D. of the monocarboxymethylated carnitine acetyltransferase, obtained after treatment of the enzyme with bromoacetyl-L-carnitine and the subsequent spontaneous hydrolysis of the carboxymethyl-enzyme-carnitine ester bond (J. F. A. Chase, P. K. Tubbs & R. N. Perham, unpublished work), shows a value of $[M']_{233}$ intermediate between that of the free enzyme and that of carnitine-saturated enzyme. No O.R.D. change is seen when carboxymethyl-enzyme is exposed to L-carnitine. $[M']_{233}$ for enzyme inhibited by prolonged exposure to CoA (Fig. 4) has a similar intermediate value and is also unaffected by the presence of L-carnitine. In each of these cases it would seem either that the inhibited enzyme form has lost the ability to bind carnitine or that the carnitine-induced configurational change characteristic of the native enzyme can no longer take place.

The mechanism by which carnitine acetyltransferase is inactivated on prolonged incubation with CoA is not known. One possibility, that CoA

becomes covalently attached to the enzyme protein by formation of a disulphide link to a cysteine residue, is rendered unlikely by the failure of dithiothreitol to reverse such inhibition.

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