STABILITY OF DICYCLOHEXYLCARBODIIMIDE IN AN AQUEOUS MEDIUM. THE EFFECT OF MITOCHONDRIAL PHOSPHOLIPIDS

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The stability of N,N'-dicyclohexylcarbodiimide (DCCD) was analyzed with respect to the use of DCCD as a specific chemical modifier of membrane-bound enzymes. The disappearance of DCCD from the sucrose–Tris medium obeyes the pseudo-first-order kinetics the rate constant (k_d) of which is pH-dependent ($k_d = 12{\cdot}0 \,.\, 10^{-3}$ min^{-1} at pH 6·0, and $k_d = 1{\cdot}6 \,.\, 10^{-3}$ min^{-1} at pH 9·0, respectively). However, the rate of the total [^{14}C]DCCD binding to mitochondrial membrane proteins is not markedly influenced by the change of pH (6·0—9·0). The rate of DCCD disappearance is enhanced in the presence of mitochondrial phospholipids. It is concluded that the rapid equilibration of DCCD between the phospholipid and water phase, the reactivity of DCCD with phospholipids, H^+-stimulated hydration of DCCD to dicyclohexylurea and the sorption of DCCD to the test tube must be considered when studying the interaction of DCCD with membrane-bound enzymes.

Because of their reactivity with various organic functional groups, carboxyl groups in particular[1], carbodiimides represent an important class of versatile reagents in organic synthesis. They have become widely used also as modifiers of some functional groups of proteins. A method was described for a quantitative determination of carboxyls in water-soluble protein with the use of polar carbodiimides[2]; and apolar N,N'-dicyclohexylcarbodimide (DCCD) was used for modification of essential residues of several enzymes localised on biomembranes[3–5]. In the field of bioenergetics, DCCD appeared to be a very useful inhibitor of proton-translocating ATPases of mitochondria, chloroplasts and bacteria, due to the modification of a specific carboxyl group deeply buried in the hydrophobic membrane environment (for a review see ref.[6]).

A serious drawback inherent in the use of DCCD to study membrane bound enzymes is the uncontrolled rate of disappearance of the carbodiimide from the medium during experiments. This decrease of the actual DCCD concentration might be caused by hydration of DCCD (ref.[1]) in aqueous solution resulting in the formation of N,N'-dicyclohexylurea or in possible side reaction of DCCD with different mem-

brane constituents. In the present report which was evoked by the kinetic analysis of the DCCD-ATPase interaction in mitochondria[7], attempts were made to evaluate the rate of DCCD disappearance in a sucrose–Tris medium, *i.e.* under the conditions mostly used in experiments with biomembranes. To ascertain the role of a major lipid constituent of the membranrs, the influence of mitochondrial phospholipids was also analysed.

MATERIAL AND METHOD

[^{14}C]DCCD was synthesized from [^{14}C]urea (32 mCi per mol) *via* N,N'-dicyclohexylurea[8] and stored as an ethanolic solution at −20 C. Both the purity of the preparation, as analysed by gas–liquid chromatography, and inhibitory effect on mitochondrial ATPase activity (ref.[7]) were equal in comparison with that of [^{14}C]DCCD commercially available (CEA, France). [^{14}C]DCCD binding to mitochondrial proteins was estimated as previousoy described[9]. Mitochondrial phospholipids were isolated and purified according to Rouser and Fleischer[10] and Radin[11]. Phospholipids were dissolved in chloroform and stored at −20°C. Prior to their further use, chloroform was evaporated under nitrogen and phospholipids were sonicated in the presence of the sucrose–Tris medium until the mixture clarified. Phospholipid phosphorus was determined according to Bartlett[12]. Protein was estimated according to Lowry and coworkers[13] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

When the fate of DCCD present at relatively low concentrations in an aqueous medium (as used *e.g.* in experiments with mitochondria) is studied, a spectrophotometric determination of DCCD concentration[1] or a chromatographic method[14] could not be used because of their low sensitivity. We attempted, therefore, to evaluate the rate of DCCD disappearance indirectly utilizing the fact that DCCD reacts covalently with some proteins of mitochondrial membrane whereas dicyclohexylurea does not[14]. In a typical experiment, [^{14}C]DCCD was preincubated in a sucrose–Tris medium and, at time intervals, aliquots were transferred to the suspension of mitochondria and the time course of the [^{14}C]DCCD binding to mitochondria was measured. As follows from Fig. 1, [^{14}C]DCCD added to the aqueous medium (pH 7·0) gradually disappeared during 6 h of the incubation resulting in decreasing rates of [^{14}C]DCCD binding.

The initial velocity of [^{14}C]DCCD binding (v_0) should follow the pseudo-first-order kinetics

$$v_0 = k \,.\, c_{\mathrm{DCCD}}\,, \qquad (1)$$

where k is the pseudo-first-order rate constant of [^{14}C]DCCD-protein interaction and c_{DCCD} is [^{14}C]DCCD concentration. Thus, the exponential decrease, as shown in the semilogarithmic plot in Fig. 2, indicates that the DCCD degradation follows the first-order kinetics, as previously demonstrated for hydration of aromatic carbo-

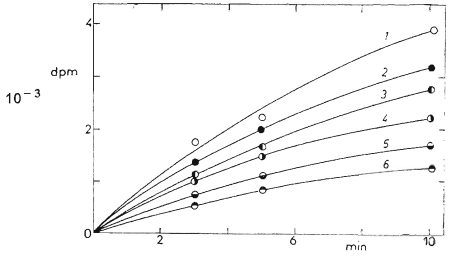

FIG. 1

Effect of the preincubation of [^{14}C]DCCD in the aqueous medium on [^{14}C]DCCD binding to mitochondria. To 5 ml of the medium containing 0·25M sucrose, 50 mM Tris-Cl (pH 7·0), 10 µl of ethanolic solution of [^{14}C]DCCD (6 µmol . l^{-1}) were added and the mixture was incubated in a glass test tube at 28°C. *1* 20 min, *2* 60 min, *3* 120 min, *4* 180 min, *5* 270 min and *6* 360 min after [^{14}C]DCCD addition 450 µl-aliquots were transferred in to separate test tubes, mixed with 50 µl of mitochondria (0·5 mg protein in 0·25M sucrose) and incubated further at 28°C. At indicated time intervals 100 µl-aliquots of the mitochondrial suspension were taken to estimate [^{14}C]DCCD binding

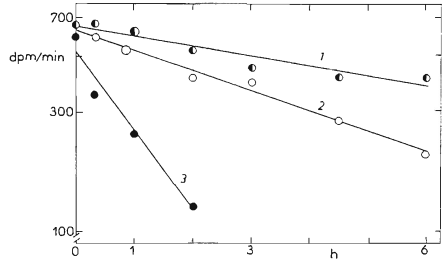

FIG. 2

The semilogarithmic plot of the initial velocity (v_0) of [^{14}C]DCCD binding to mitochondrial *versus* time of preincubation of carbodiimide in the aqueous medium at various pH. Initial velocities 2 at pH 7·0 were obtained from curves in Fig. 1. The velocities *1* at pH 9·0 and *3* at pH 6·0 were obtained as in Fig. 1. For the preincubation at pH 6·0, Tris was replaced with 50 mM morpholinopropanesulfonic acid

diimides[1]. The experimental points in Fig. 2 were therefore fitted by the standard least square procedure to the relation

$$v_0 = (v_0)_{t=0} \exp(-k_d \cdot t) , \qquad (2)$$

where k_d is the first-order rate constant of DCCD degradation. The values of parameters giving the best fit are presented in Table I. Whereas the value of the product $k \cdot c_{DCCD}$ (*i.e.* v_0; see equation (*1*)) does not depend on pH, the value of k_d is strongly pH dependent (Table I). DCCD thus belongs to the class of acid-sensitive carbodiimides[1]. N,N-dicyclohexylurea formed by hydration of DCCD in water via a protonated intermediate[1] represents most probably the degradation product.

Referring to the interaction of DCCD with biomembranes the lower [^{14}C]DCCD binding to the bacterial[15] and mitochondrial membranes as a result of acidification could be casued mainly by the enhanced hydration of DCCD in aqueous phase. On the other hand the reactivity of the target groups in the membrane towards DCCD does not seem to be influenced by the change in pH, reflecting probably the fact, that the major part of DCCD-reactive sites is localized in the hydrophobic membrane environment.

In addition to the hydration of DCCD in aqueous medium, binding of N,N'-carbodiimide to the walls of glass test tube is involved, as shown by an about 20% decrease of radioactivity occurring during the first hour of incubation at various pH tested. The sorption of DCCD is considerably higher when polypropylene Eppendorf tubes are used (about 50% of DCCD added; conditions similar as in Fig. 1) and the rate of DCCD disappearance is accelerated by about three times, as compared with the incubation in glass (not shown).

The presence of mitochondrial phospholipids in the aqueous phase highly stimulated the disappearance of [^{14}C]DCCD (Fig. 3). This was again evident from the rate

TABLE I

The initial velocities of binding (v_0) and the first-order rate constants of [^{14}C]DCCD degradation (k_d) at various pH. Data from Fig. 2 were used (see text)

pH	v_0 dpm bound. min^{-1}	$k_d \cdot 10^3$ min^{-1}
6·0	523 ± 70	12·0 ± 2·0
7·0	639 ± 30	3·1 ± 0 2
9·0	672 ± 30	1·6 ± 0 2

of binding of [^{14}C]DCCD to mitochondrial proteins which decreased approximately four times, in comparison with simple aqueous medium (Fig. 1). This was clearly manifest at the shortest incubation intervals tested when the hydration of DCCD was still negligible (Fig. 1 and 3). In addition, as shown in Fig. 3, all [^{14}C]DCCD gradually disappeared within 1 h of the preincubation with phospholipids. Therefore it is suggested that the disappearance of [^{14}C]DCCD is accelerated because: (*a*) the amount of [^{14}C]DCCD available for the reaction with mitochondria is decreased by a rapid equilibration of the apolar carbodiimide between water and added phospholipids; and (*b*) carbodiimide has been consumed in the chemical reaction with some reactive group of added phospholipids (*e.g.* condensation of carboxyl of phosphatidylserine with some nucleophilic group; ref.[1]). If only the first possibility existed, there should be some lower but still significant time-dependent binding to mitochondria after 1 h of the incubation (Fig. 3).

It is inferred that phospholipids might play a dual role in influencing the interaction of DCCD with membrane bound proteins: (*a*) phospholipids form an hydrophobic environment surrounding the reactive proteins where DCCD concentrates, and the rate of the DCCD proteins interaction is thus increased[7]; (*b*) concomitantly the actual concentration of DCCD around the protein site is decreased by the competing reaction of DCCD with phospholipids. In fact the antagonistic effect of phospholipids on the DCCD-induced inhibition of the mitochondrial ATPase activity has already been described[16]; and unstable products of the reaction of [^{14}C]DCCD with phospholipids in the mitochondrial membrane were also detected[17]. The observed rate of [^{14}C]DCCD disappearance from the medium (Fig. 3) could partially explain why only 50% of the added [^{14}C]DCCD remained bound to the mitochondrial membrane after the completion of the reaction (after 2 h of incubation; ref.[7,9]).

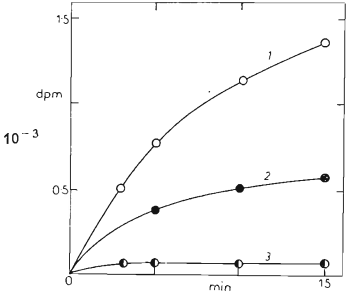

FIG. 3

The effect of mitochondrial phospholipids on the rate of decrease of [^{14}C]DCCD] concentration in the aqueous medium. The experiment was performed as in Fig. 1. The medium (pH 7·0) contained in addition sonicated mitochondrial phospholipids (15 μg phospholipid phosphorus per ml). *1* 1 min, *2* 20 min and *3* 60 min after [^{14}C]DCCD addition aliquots of the mixture were removed to follow the rate of [^{14}C]DCCD binding to mitochondria

On the basis of the results presented here, it is concluded that during the reaction of DCCD with membrane-bound enzymes the actual concentration of DCCD is decreased as a consequence of several processes: (*a*) the sorption of DCCD to the test tube walls; (*b*) the H^+-activated hydration of DCCD to dicyclohexylurea; (*c*) the interaction of DCCD with membrane phospholipids. These processes must be taken into account when kinetic experiments with DCCD are performed[7], when DCCD-sensitivity of various preparations containing ATPase (mitochondrial isolated enzyme) differing in the content of phospholipids is compared or when specific labelling of [^{14}C]DCCD-binding sites is investigated in the presence of various amounts of phospholipids[18].

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