The binding of tyrosine hydroxylase to negatively charged lipid bilayers involves the N-terminal region of the enzyme

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Abstract Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of catecholamines. We have studied the association of recombinant human TH with model membranes by using either liposomes or silica gel beads coated with single phospholipid bilayers (TRANSIL®). The use of TRANSIL beads has allowed the determination of apparent dissociation constants (K_d) for the binding of the enzyme to negatively charged bilayers (K_d = 230–380 μM, at pH 6.0–7.0). Binding to the bilayers is accompanied by a decrease in enzyme activity. Proteolysed forms of the enzyme show decreased binding affinity and two putative amphipathic N-terminal α-helices are proposed to be involved in membrane binding. As seen by circular dichroism, binding to the bilayer does not seem to induce significant changes on the secondary structure content of the enzyme, but α-helical structures appear to be stabilized against thermal denaturation in the membrane-bound state. Thus, amphitropism, a mechanism that regulates the function of peripheral proteins by weak binding to membrane lipids, may add to the factors that regulate both the activity and the stability of TH.

Key words: Tyrosine hydroxylase; Membrane binding; Phospholipid-coated bead; Limited proteolysis; Amphipathic α-helix; Circular dichroism

1. Introduction

Tyrosine hydroxylase (TH, EC 1.14.16.2) catalyzes the hydroxylation of L-Tyr to L-DOPA, which is the rate-limiting reaction in the biosynthesis of catecholamines. The enzyme is mainly expressed in dopaminergic and noradrenergic neurons in the brain, in the peripheral sympathetic nervous system, and in chromaffin cells in the adrenal medulla [1]. In brain, TH seems to exist in two distinct forms, a soluble and a membrane-associated form [2]. The distribution of both forms seems to be region-specific with the soluble form predominating in the substantia nigra and locus coeruleus, while in nerve endings or axons the enzyme seems to be associated with neurotubules and membranes [1]. In adrenal chromaffin cells TH is mostly found in the cytoplasm, but considerable evidence has also been presented on its association with the membrane of the chromaffin granule [3–5]. The association seems to be weak and not to involve the core of the enzyme, since an active soluble TH form is released from the membrane by limited proteolysis [6,7]. However, it has also been reported that TH exists as an integral component of granule membranes [8]. Although it has been argued that fractionation or fixation procedures may result in the redistribution of cellular components, including TH [9], it is now accepted that the protein exists in soluble and membrane-associated forms. Thus, TH seems to be an amphitropic protein [10,11]. Amphitropic proteins are found as both soluble and peripheral membrane proteins, their interaction with membranes being weak ($K_d > 1$ μM), and usually their function is modulated by membrane binding. It has been proposed that binding of TH to the storage secretory granules might play a role in coordinating TH activity and catecholamine release [5], although the physico-chemical basis and physiological significance of the association of TH with membranes is still unknown.

In this work we have characterized the interaction of recombinant human TH isoform 1 (hTH1) with phospholipid bilayers of defined composition, as well as the effects of membrane association on the activity and the conformational stability of the enzyme. We have used both liposomes and TRANSIL®, a solid supported lipid membrane system [12]. TRANSIL beads have characteristics typical of the lipid bilayer of biological membranes and have the advantage of being easily separated from the aqueous phase by sedimentation [12,13]. The use of TRANSIL has allowed us to determine the dissociation constants for the interaction of full-length and proteolysed forms of hTH1 with membranes of different phospholipid composition. As found with several amphitropic proteins [11], putative amphipathic N-terminal α-helices seem to be involved in the binding of TH to the membrane.

2. Materials and methods

2.1. Materials

(6*R*)-5,6,7,8-Tetrahydrobiopterin (BH_4) was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). Egg phosphatidylcholine (PC, grade 1) and 1,2-dioleoylphosphatidylglycerol (DOPG) were purchased from Sigma (St. Louis, MO, USA). Bovine brain L-3-phosphatidylserine (BBPS) was from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ, USA). TRANSIL® beads (29 ± 2.5 μm particle

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Abbreviations: BBPS, bovine brain L-3-phosphatidylserine; CD, circular dichroism; DEPC, dielaidoylphosphatidylcholine; DOPG, 1,2-dioleoylphosphatidylglycerol; hTH1, recombinant human tyrosine hydroxylase isoform 1; PC, phosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; SUV, small unilamellar vesicles; TH, tyrosine hydroxylase

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size; 400 nm pore size; 12 m^2/g phospholipid) coated with either egg PC (TRANSIL PC), PC:DOPG (3:2 molar ratio; TRANSIL PC/DOPG) or dielaidoylphosphatidylcholine (DEPC):1-palmitoyl-2-oleoylphosphatidylserine (POPS) (4:1 molar ratio; TRANSIL DEPC/POPS) were obtained from Nimbus GmbH (Leipzig, Germany).

2.2. Enzyme purification and assay

Recombinant human TH, isoforms 1 and 4 (hTH1 and hTH4), were expressed in *Escherichia coli* and purified to homogeneity by heparin-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) chromatography as previously described [14]. Equimolar amounts of ferrous ammonium sulfate per enzyme subunit were added to convert the iron-free apoenzyme into holoenzyme [15]. The enzyme is a tetramer and the molar concentration is given per enzyme subunit. Assay for TH activity was performed at 25°C by the method of Reinhard et al. [16] using an incubation mixture containing 100 mM Na-HEPES pH 7.0, 25 μM L-[3,5-^{3}H]tyrosine (Amersham Pharmacia Biotech, UK), 0.5 mg/ml catalase and 100 μM Fe(II)SO_4. The enzyme was preincubated for 3 min in this mixture before the reaction was started by addition of 500 μM BH_4 and 5 mM dithiothreitol as the tetrahydropterin-regenerating agent. The reaction was stopped after 3 min by the addition of a slurry of activated charcoal in 1 M HCl. After centrifugation, an aliquot of the supernatant was counted in a scintillation counter.

2.3. Limited proteolysis by trypsin

Limited tryptic digestion of hTH1 (1.5 mg/ml) was performed at 30°C in 20 mM Na-HEPES, 0.1 M NaCl, pH 7.0 at a trypsin:hTH1 ratio of 0.00075:1 (w:w). The reaction was stopped at the indicated time period by the addition of 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone. The products of proteolysis were analyzed by high resolution size exclusion chromatography as described [17] and by SDS–PAGE in 10% (w/v) polyacrylamide gel [18].

2.4. Preparation of liposomes

Dried lipid films of specified phospholipid composition were dispersed in 20 mM Na-HEPES, 0.1 M NaCl, pH 7.0 in a sonicating bath during 1 h at 4°C. A highly homogeneous preparation of small unilamellar vesicles (SUV), also referred to as liposomes, was obtained. The diameter of the SUV was 40 ± 5 nm, as seen by electron microscopy and quasi-elastic light scattering using a Malvern Zetasizer (Malvern, UK) and by gel filtration chromatography on Superdex 200 FPLC column (1 × 30 cm) (Amersham Pharmacia Biotech). Electron microscopy revealed that the liposomes were unilamellar.

2.5. Gel filtration chromatography

SUV preparations (0.5–5 mM in phospholipid) in 20 mM Na-HEPES, 0.1 M NaCl, pH 7.0 were mixed with hTH1 (46 μM subunit) at 20°C for 30 min, and then the SUV were separated from unbound hTH1 on a calibrated Superdex 200 (1 × 30 cm) gel filtration column (Amersham Pharmacia Biotech) eluted at 0.5 ml/min with the same buffer. Fractions of 0.5 ml were collected and the protein content was determined spectrophotometrically at 280 nm ($\varepsilon^{1\%} = 10.4$ cm^{-1}) [19] and by the Bradford method [20] while phospholipids were determined as inorganic phosphorus using the modified procedure [21] of the malachite green assay [22].

2.6. Binding of hTH1 to TRANSIL beads

TRANSIL silica gel beads coated with a phospholipid bilayer of desired composition (TRANSIL PC, TRANSIL PC/DOPG or TRANSIL DEPC/POPS) were equilibrated in 20 mM Na-HEPES, 0.1 M NaCl, pH 6.0 or 7.0 by repeated washing and sedimentation. The binding of hTH1 was performed by incubating the TRANSIL dispersions (at the indicated final lipid concentration) with the indicated amounts of hTH subunit in the same buffer. The solutions (final volume 50 μl) were incubated at room temperature for 30–60 min, as indicated, and the beads were maintained in suspension by gentle shaking. In a reference sample, TRANSIL beads were replaced by buffer. After centrifugation (1 min at 1000 × *g*), the protein content in the supernatants was analyzed by SDS–PAGE in 10% (w/v) polyacrylamide gels [18]. The SDS–PAGE step was introduced to increase the sensitivity of the detection of protein in the supernatants (detection limit 0.05 μg). Gels were stained with Coomassie brilliant blue, scanned and the staining density of the bands was measured at $A_{633\,nm}$ using the Phoretix 1D analysis software (Nonlinear Dynamics Ltd, 1996). A linear correlation was found between the $A_{633\,nm}$ and the amount of protein applied to the gel from 0.05 to 2 μg. Data were further analyzed using the SigmaPlot program (Jandel Scientific).

The partition coefficients (K_p) or effective association constants (K_a), obtained as described [12], were defined as

$$K_p = \frac{[P]_{bound}}{[L][P]_{free}} \quad (1)$$

and

$$\frac{[P]_{bound}}{[P]_{total}} = \frac{K_p[L]}{(1 + K_p[L])} \quad (2)$$

$[P]_{bound}$ and $[P]_{free}$ being the concentration of membrane-bound and free protein, respectively, $[P]_{total} = [P]_{bound} + [P]_{free}$, and [L] is the concentration of lipid. The effective dissociation constant ($K_d = 1/K_p$) corresponds to the concentration of accessible lipid at which 50% of protein is bound.

2.7. Circular dichroism (CD)

CD measurements were performed on a Jasco J-810 spectropolarimeter equipped with Jasco PTC-423S Peltier element for temperature control. Enzyme samples were prepared in 20 mM Na-phosphate, 0.1 M NaCl, pH 7.0, and placed in quartz cells with a path length of 1 or 5 mm. The hTH1 concentration was 5 μM subunit, and the phospholipid concentration was 466 or 933 μM, as indicated, and under these experimental conditions it was estimated that about 80–85% of the protein was associated with membranes (see below). References (buffer or buffered liposome solution) were routinely subtracted from the original spectra. Estimation of secondary structure elements was performed by the CDNN program, which applies a backpropagation neural network model for the quantitative analysis of protein far-UV CD spectra [23]. Protein thermal denaturation was monitored by following the change in ellipticity at 222 nm in the range 25–70°C, with a scan rate of 0.7°C/min. Analysis of the data and determination of T_m values were performed by noise reduction and differentiation of the curves using the Standard Analysis program provided with the instrument.

3. Results and discussion

3.1. hTH1 binding to liposomes and to TRANSIL beads

The association of hTH1 with liposomes was first studied by incubating the enzyme (7 μM) for 30 min at 20°C with SUV (500 μM phospholipid) composed of either PC, PC:DOPG (1:1) or PC:BBPS (1:1) at pH 7.0. The incubation mixtures were separated by gel filtration chromatography. The enzyme did not bind to SUVs composed of PC, but it did to negatively charged SUV of PC:DOPG or PC:BBPS and the resulting fractions were analyzed for protein and phospholipid content (see representative gel filtration profiles in Fig. 1), indicating approx. 85% binding at these conditions. The peak centered at around fraction 25, with an elution volume of 12.5 ml (Fig. 1B), corresponds to SUV with a diameter of 40 ± 5 nm, as seen by quasi-elastic light scattering, and upon molecular mass standardization of the gel filtration column (thyroglobulin – 670 kDa – shows the same elution volume). The position of this peak was the same in the absence or in the presence of hTH1. Control incubations performed with bovine serum albumin showed no binding of this protein to SUV composed of either PC:DOPG or PC:BBPS, indicating the absence of non-specific protein association with the vesicles. The association of hTH1 with phospholipid bilayers was also studied using TRANSIL, a silica gel coated with a lipid bilayer, with SDS–PAGE-based protein detection [12,13]. The use of this approach allows the estimation of the partition coefficients between the aqueous phase and the membrane for hTH1, since the separation of the membrane-bound

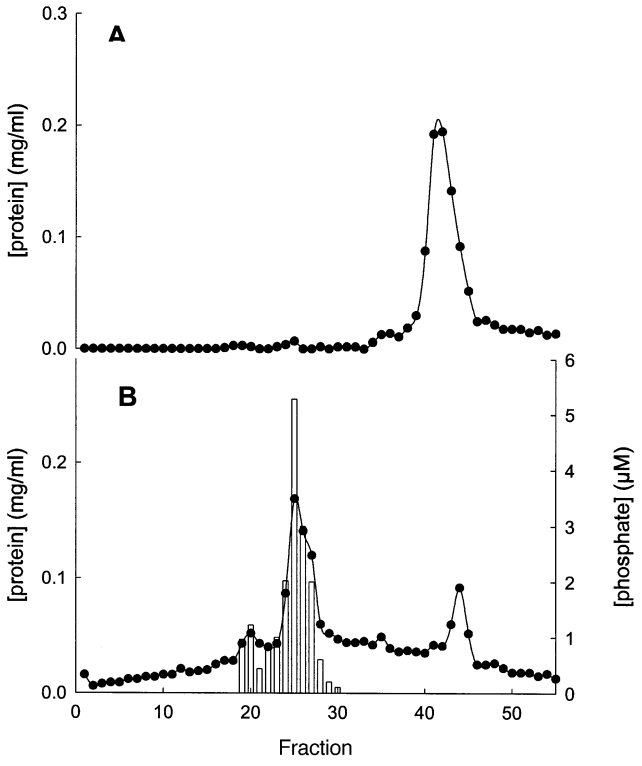

Fig. 1. Binding of hTH1 to liposomes analyzed by gel filtration chromatography. A: Elution of hTH1 in the absence of liposomes on a Superdex 200 (1×30 cm) gel filtration chromatography column at 0.5 ml/min, pH 7.0. B: Elution of a mixture of vesicles composed of PC:DOPG (1:1) and recombinant hTH1 (30 min incubation at 20°C previous to chromatography). Fractions (0.5 ml) were assayed independently for protein (●) and phospholipid concentration (bars).

and free protein required a simple centrifugation step (5 min, 1000×*g*) keeping nearly equilibrium binding conditions. Moreover, the sensitivity of the method permits the assessment of binding at several phospholipid concentrations. In spite of the different diameter size of the TRANSIL beads and SUVs, it has been shown for some peripheral proteins that for a given phospholipid composition the membrane partition coefficients for the proteins estimated using TRANSIL beads are in agreement with those obtained using the vesicles [12]. We investigated the binding affinity of hTH1 for TRANSIL beads coated with three relevant phospholipid compositions, i.e. TRANSIL PC (neutral bilayer) and TRANSIL PC/DOPG or TRANSIL DEPC/POPS (negatively charged bilayers). The enzyme did not bind to TRANSIL PC (Table 1), but it did to both types of negatively charged beads at neutral pH (Fig. 2A,B and Table 1). For these vesicles the K_d values decreased when the binding assay was performed at pH 6.0, especially for DEPC/POPS, while no binding to TRANSIL PC was detected at either pH (Table 1). Although the instability of hTH1 precludes measurements at pH < 6.0, the above data suggest that electrostatic interactions might be involved in the association process. The K_d values measured with TRANSIL beads correspond to binding constants (K_a) from 2×10^3 to 4×10^3 M^{-1}, well within the range reported for the binding of amphitropic proteins to negatively charged membranes ($K_a < 10^7$ M^{-1}) [11]. As an example, proteins that associate with membranes via a myristoyl electrostatic switch show K_a values of 10^3–10^4 M^{-1}, binding constants characteristic of a weak binding ($\Delta G = 6.5$–7.8 kcal/mol) [24]. There has been a reasonable agreement between the binding constants measured in vitro and the membrane affinity assessed in vivo [11], although care must be taken to extrapolate parameters obtained with model systems to the real situation in the cell due to the complex thermodynamics of binding.

3.2. *Effect of hTH1 association to the membranes on the enzyme activity*

Previous studies by Morita et al. have shown an inhibition of the TH activity upon peripheral association of the enzyme with isolated chromaffin granula membranes [4]. On the other hand, Kuhn et al. reported that the integral form of the enzyme shows a higher specific activity than the cytoplasmic form [8]. We measured the effect of the association of hTH1 to SUVs made of either PC alone, PC:BBPS (1:1) or PC:DOPG (1:1) on the enzyme activity. In agreement with the binding assays, the presence of liposomes composed of PC at concentrations up to 3.5 mM phospholipid did not have a significant effect on the enzyme activity, while liposomes made of either PC:BBPS or PC:DOPG had an inhibiting effect. Fig. 2C shows a representative inhibition curve with liposomes of PC:DOPG. Thus, at a phospholipid concentration higher than 1 mM (lipid/hTH1 subunit molar ratio ranging from 85 to 300), the activity was reduced to 30% of that found for free hTH1 in the absence of liposomes. The concentration of phospholipid causing a 50% inhibition (IC_{50}) of the activity was estimated to be about 200 μM both for PC:BBPS and PC:DOPG liposomes. This reduction of the activity may be caused by a decreased mobility of the bound protein or a steric hindrance of the active site. It has been argued by Kuhn et al. [8] that the partial inactivation of the membrane associated bovine TH observed by Morita et al. [4] was due to the presence of inhibitors of TH in the chromaffin granule membranes. Nevertheless, it has also been shown that TH isolated from the cytoplasmic fraction of cell/tissues yields a high amount of enzyme forms complexed with catecholamines [25–27], which reversibly inhibit enzyme activity. The binding of catecholamines to the soluble as well as to the membrane associated enzyme may thus complicate the interpretation of regulatory modifications of the enzyme. It has earlier been shown that the true conformational and catalytic effects of covalent or other regulatory modifications of TH are better studied in recombinant forms of the enzyme which are devoid of any bound catecholamines [27,28], as is the case in the

Table 1
Dissociation constants (K_d) for the interaction of hTH1 with model membranes using TRANSIL beads

Enzyme	pH	TRANSIL PC/DOPG K_d (μM)[a]	TRANSIL DEPC/POPS K_d (μM)[a]	TRANSIL PC
hTH1	6.0	375 ± 8	228 ± 18	No binding
hTH1	7.0	415 ± 21	306 ± 24	No binding

[a]hTH1 (1 μM subunit) and TRANSIL beads (up to 3 mM phospholipid) were incubated 30 min at room temperature. Similar K_d values were obtained using a 10-fold higher protein concentration and an incubation time of 60 min.

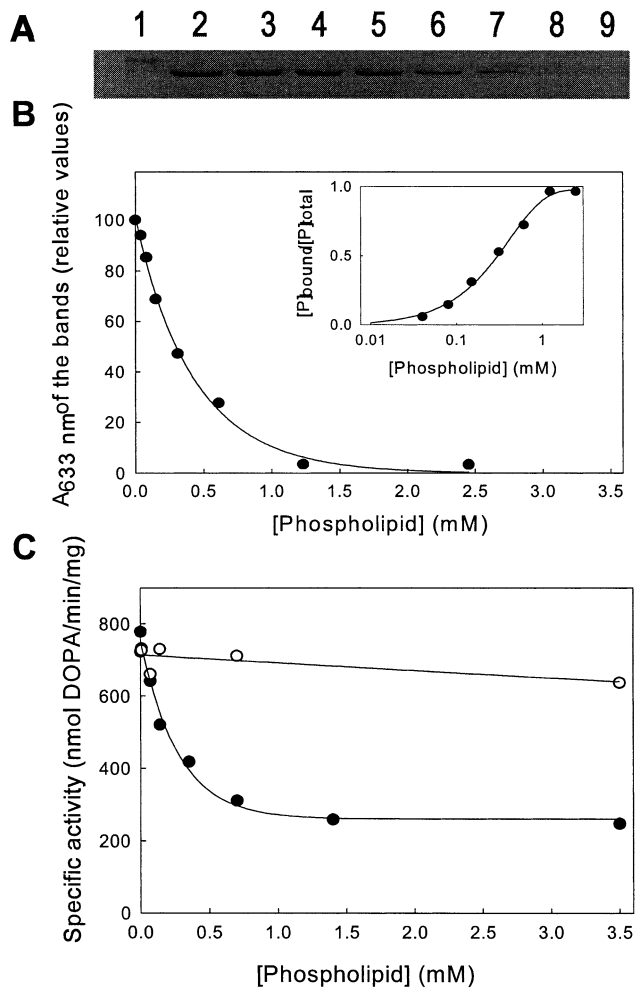

Fig. 2. Binding of hTH1 to phospholipid-coated beads and effect of membrane binding on the enzyme activity. Different amounts of TRANSIL beads coated with PC:DOPG (3:2) were incubated with hTH1 (72 μg/ml) at pH 7.0 for 30 min at room temperature under end-over-end rotation. The beads were then centrifuged and the protein remaining in the supernatant was resolved on SDS–PAGE (A). Phospholipid concentrations in μM were 0 (lane 2), 40 (lane 3), 80 (lane 4), 150 (lane 5), 310 (lane 6), 610 (lane 7), 1230 (lane 8) and 2450 (lane 9). Bovine serum albumin (66.2 kDa) (lane 1). B: Quantification of the data presented in A by densitometric scanning of the staining density of the bands in the gels (see Section 2) and calculation of the effective K_d (inset). C: The activity of hTH1 was measured under standard experimental conditions (pH 7.0, 25°C) in the presence of increasing amounts of liposomes made of PC (○) or PC:DOPG (1:1) (●), up to 3.5 mM in lipid.

present study. Thus, the use of hTH1 and phospholipid membranes of defined composition gives further credit to the finding that the specific activity of the enzyme is reduced upon binding to negatively charged lipid vesicles. This reduction may be caused by a decreased mobility of the bound protein or a steric hindrance of the active site. It is therefore tempting to propose that variations in the lipid composition of the membrane may modulate the membrane binding capacity and the activity of hTH in different cell types (neurons or chromaffin cells) or in different areas of the brain.

3.3. The role of the N-terminal region on membrane binding

The involvement of electrostatic interactions in the binding of hTH1 to bilayers is indicated by the fact that no binding occurs in the absence of anionic lipid and by the slight decrease in K_d with decreasing pH (Table 1). Morita et al. [5] investigated the association of TH with isolated chromaffin granule membranes and also found that the interaction was modulated by ionic factors. To further investigate the electrostatic requirement and the involvement of different protein domains on the interaction of hTH1 with membranes, the enzyme was subjected to limited tryptic digestion and we studied the association of the truncated forms with lipid bilayers. Limited proteolysis results in the cleavage of the 56 kDa full-length enzyme subunit into species of 54 kDa, 52 kDa and 47 kDa (Fig. 3A). As seen by high resolution size exclusion chromatography these truncated forms were tetrameric, indicating that they still contain the C-terminal α-helical motif [29]. Moreover, in studies with rat TH, which shows high homology with hTH1, the 52 kDa species have been shown by N-terminal sequencing to result from the cleavage of the full-length protein at various Arg residues at the N-terminal, from position 33 to 49 [30]. These Arg residues are conserved in hTH1. Even though various chromatographic procedures were undertaken, no further purification of these two proteolysed forms could be achieved, possibly due to the combination of both truncated forms in the tetrameric enzyme. However, by controlling the time of incubation by trypsin and the trypsin:hTH1 ratio, proteolysed forms containing mostly (80% of the total protein) the 52 kDa truncated form could be prepared. No measurable binding of the truncated forms could be observed to either TRANSIL PC (data not shown) or TRANSIL PC/DOPG (Fig. 3B).

We analyzed the distribution of the charged residues within

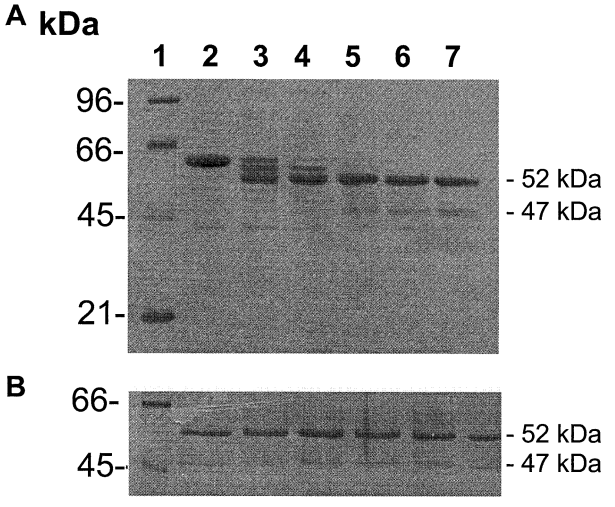

Fig. 3. Effect of enzyme deletions on the binding of hTH1 to phospholipid coated beads. A: The time course of limited proteolysis of hTH1 by trypsin. The enzyme (1.5 mg/ml) was incubated at 30°C, pH 7.0, at a trypsin:hTH1 ratio of 0.00075:1 (μg:μg) for 0 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 6 (lane 6) and 8 (lane 7) min and the reaction was stopped with trypsin inhibitors (see Section 2). Molecular weight standard proteins (lane 1). B: Proteolysed hTH1 (sample shown in A, lane 6), containing largely the 52 kDa truncated form, was incubated with different amounts of TRANSIL beads coated with a PC:DOPG (3:2) bilayer, at pH 7.0 for 30 min at room temperature and the binding was analyzed by SDS–PAGE. The phospholipid concentrations in μM are 0 (lane 2), 40 (lane 3), 80 (lane 4), 150 (lane 5), 310 (lane 6) and 1230 (lane 7). Bovine serum albumin (66.2 kDa) and ovalbumin (45 kDa) (lane 1).

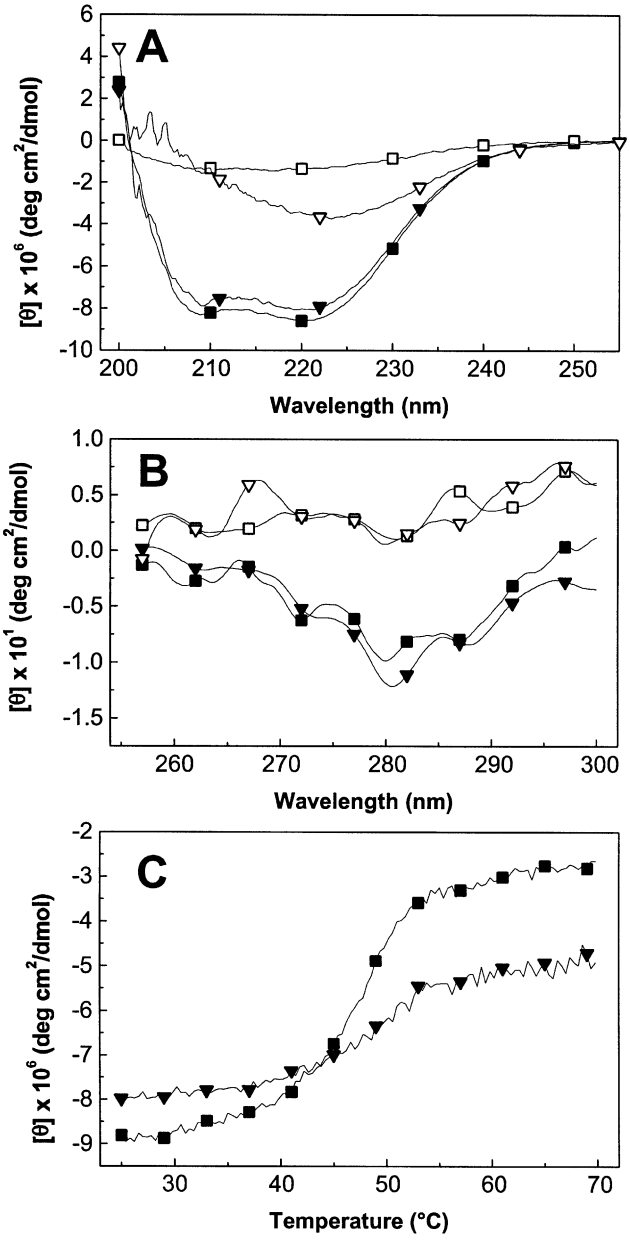

Fig. 4. CD spectra of hTH1 in solution and bound to liposomes. Spectra were recorded at pH 7.0, 25°C with 5 μM hTH1 subunit and lipid concentration of 466 μM. CD spectra measured at a lipid concentration of 933 μM were similar. Far-UV CD spectra (A), near-UV CD spectra (B) and CD-monitored thermal denaturation (C) measured as molar ellipticity ([θ]) changes at 222 nm recorded at a scan rate of 0.7°C/min for free hTH1 (■) and hTH1 bound to liposomes of PC:BBPS (1:1) (▼). Spectra were also taken for hTH1 denatured by heating the enzyme up to 70°C (during the temperature scan) in the absence (□) and presence of PC:BBPS (1:1) liposomes (▽).

the N-terminal region and searched for putative secondary structure elements that this region could host. No structural information is available for the N-terminal 1–65 residues in hTH1 [31]. Based on predictive methods [32], two putative helical segments are predicted for this sequence. The first helix comprises residues 15–30 and shows a positively charged face containing Lys24 and the second longer helical segment spans residues 37–58 and also shows a spatial distribution of the charged residues, with Arg38, Arg46 and Arg49 at one face of the helix. The total or partial cleavage of these helices by trypsin could be responsible for the lack of interaction of the truncated fragments with negatively charged membranes and is in agreement with earlier studies showing the isolation of active truncated forms of the enzyme from bovine adrenal medulla membranes by limited proteolysis [6,7].

3.4. Effect of membrane binding on the conformation and stability of hTH1

To further explore the effect of membrane binding on the conformational properties of hTH1 we applied CD spectroscopy. As previously reported for the recombinant rat enzyme [33], the far-UV CD spectrum of hTH1 shows minima at 208 and 222 nm (Fig. 4A), typical of a largely α-helical structure. The amount of α-helical structure estimated using a neural network procedure [23] was 42 ± 2% and correlates well with that obtained from infrared spectroscopic studies [15] and crystallographic analyses of the catalytic domain [29]. Moreover, CD studies with the catalytic domain of rat TH have associated the negative signal at about 280 nm in the near-UV CD spectrum of hTH1 (Fig. 4B) to tryptophanyl residues [33]. The presence of liposomes at saturating concentrations with respect to the association of hTH1, i.e. phospholipid concentration >1 mM, hampers the acquisition of CD spectra with satisfactory signal-to-noise ratio. We thus investigated the effect of membrane binding on the CD spectra and the conformational stability of hTH1 using phospholipid concentrations that ensured more than 80% binding, i.e. 466 μM and 933 μM (Figs. 1 and 4). The presence of liposomes of either PC:DOPG (1:1) or PC:BBPS (1:1) did not significantly change the far- (Fig. 4A) and near-UV (Fig. 4B) CD spectra of the protein. The thermal unfolding of free and membrane-bound hTH1 was followed by measuring the molar ellipticity at 222 nm, the benchmark for α-helix, as a function of temperature (Fig. 4C). The T_m of the denaturation transition is similar in the absence and presence of liposomes, i.e. 47°C. However, a comparison of the thermally denatured conformations shows that the membrane-bound protein retains more regular secondary structure (notably α-helix) than hTH1 in solution (Fig. 4A,C), whereas the interactions that maintain the tertiary structure are lost in both cases (Fig. 4B). The stabilization against the thermal challenge of α-helical structures in membrane-bound hTH1 suggests that the N-terminal segments of the protein that interact with the lipid bilayer are helical in the native protein. The involvement of amphipathic α-helices on the binding of amphitropic proteins to negatively charged membranes is well documented [11].

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References

[1] Kaufman, S. (1995) Adv. Enzymol. Relat. Areas Mol. Biol. 70, 103–220.
[2] Kuczenski, R. (1973) J. Biol. Chem. 248, 5074–5080.
[3] Kelner, K.L., Morita, K., Rossen, J.S. and Pollard, H.B. (1986) Proc. Natl. Acad. Sci. USA 83, 2998–3002.
[4] Morita, K., Teraoka, K. and Oka, M. (1987) J. Biol. Chem. 262, 5654–5658.

[5] Morita, K., Hamano, S. and Oka, M. (1994) Neurochem. Int. 25, 403–411.
[6] Petrack, B., Sheppy, F. and Fetzer, V. (1968) J. Biol. Chem. 243, 743–748.
[7] Shiman, R., Akino, M. and Kaufman, S. (1971) J. Biol. Chem. 246, 1330–1340.
[8] Kuhn, D.M., Arthur Jr., R., Yoon, H. and Sankaran, K. (1990) J. Biol. Chem. 265, 5780–5786.
[9] Wurzburger, R.J. and Musacchio, J.M. (1971) J. Pharmacol. Exp. Ther. 177, 155–168.
[10] Burn, P. (1988) Trends Biochem. Sci. 13, 79–83.
[11] Johnson, J.E. and Cornell, R.B. (1999) Mol. Membr. Biol. 16, 217–235.
[12] Schmitz, A.A., Schleiff, E., Rohring, C., Loidl-Stahlhofen, A. and Vergeres, G. (1999) Anal. Biochem. 268, 343–353.
[13] Frank, C., Keilhack, H., Opitz, F., Zschornig, O. and Bohmer, F.D. (1999) Biochemistry 38, 11993–12002.
[14] Haavik, J., Le Bourdelles, B., Martínez, A., Flatmark, T. and Mallet, J. (1991) Eur. J. Biochem. 199, 371–378.
[15] Martínez, A., Haavik, J., Flatmark, T., Arrondo, J.L.R. and Muga, A. (1996) J. Biol. Chem. 271, 19737–19742.
[16] Reinhard Jr., J.F., Smith, G.K. and Nichol, C.A. (1986) Life Sci. 39, 2185–2189.
[17] Knappskog, P.M., Flatmark, T., Aarden, J.M., Haavik, J. and Martínez, A. (1996) Eur. J. Biochem. 242, 813–821.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Haavik, J., Andersson, K.K., Petersson, L. and Flatmark, T. (1988) Biochim. Biophys. Acta 953, 142–156.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1984) Biochem. J. 219, 25–31.
[22] Chalvardjian, A. and Rudnicki, E. (1970) Anal. Biochem. 36, 225–226.
[23] Bohm, G., Muhr, R. and Jaenicke, R. (1992) Protein Eng. 5, 191–195.
[24] McLaughlin, S. and Aderem, A. (1995) Trends Biochem. Sci. 20, 272–276.
[25] Andersson, K.K., Cox, D.D., Que Jr., L., Flatmark, T. and Haavik, J. (1988) J. Biol. Chem. 263, 18621–18626.
[26] Andersson, K.K., Vassort, C., Brennan, B.A., Que Jr., L., Haavik, J., Flatmark, T., Gros, F. and Thibault, J. (1992) Biochem. J. 284, 687–695.
[27] Muga, A., Arrondo, J.L., Martínez, A., Flatmark, T. and Haavik, J. (1998) Adv. Pharmacol. 42, 15–18.
[28] Flatmark, T., Almås, B., Knappskog, P.M., Berge, S.V., Svebak, R.M., Chehin, R., Muga, A. and Martínez, A. (1999) Eur. J. Biochem. 262, 840–849.
[29] Goodwill, K.E., Sabatier, C., Marks, C., Raag, R., Fitzpatrick, P.F. and Stevens, R.C. (1997) Nature Struct. Biol. 4, 578–585.
[30] McCulloch, R.I. and Fitzpatrick, P.F. (1999) Arch. Biochem. Biophys. 367, 143–145.
[31] Kobe, B., Jennings, I.G., House, C.M., Michell, B.J., Goodwill, K.E., Santarsiero, B.D., Stevens, R.C., Cotton, R.G. and Kemp, B.E. (1999) Nature Struct. Biol. 6, 442–448.
[32] Rost, B., Sander, C. and Schneider, R. (1994) Comput. Appl. Biosci. 10, 53–60.
[33] Daubner, S.C., Lohse, D.L. and Fitzpatrick, P.F. (1993) Protein Sci. 2, 1452–1460.