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Inhibition of Transthyretin Amyloid Fibril Formation by 2,4-Dinitrophenol through Tetramer Stabilization

P. Raghu, G. Bhanuprakash Reddy, and B. Sivakumar[1]
National Institute of Nutrition, Indian Council of Medical Research, Hyderabad 500 007, India

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Transthyretin (TTR), a homotetrameric thyroxine transport protein found in the plasma and cerebrospinal fluid, circulates normally as a innocuous soluble protein. In some individuals, TTR polymerizes to form insoluble amyloid fibrils. TTR amyloid fibril formation and deposition have been associated with several diseases like familial amyloid polyneuropathy and senile systemic amyloidosis. Inhibition of the fibril formation is considered a potential strategy for the therapeutic intervention. The effect of small water-soluble, hydrophobic ligand 2,4-dinitrophenol (2,4-DNP) on TTR amyloid formation has been tested. 2,4-DNP binds to TTR both at acidic and physiological pH, as shown by the quenching of TTR intrinsic fluorescence. Interestingly, 2,4-DNP not only binds to TTR at acidic pH but also inhibits amyloid fibril formation as shown by the light scattering and Congo red-binding assay. Inhibition of fibril formation by 2,4-DNP appears to be through the stabilization of TTR tetramer upon binding to the protein, which includes active site. These findings may have implications for the development of mechanism based small molecular weight compounds as therapeutic agents for the prevention/inhibition of the amyloid diseases.

Key Words: **transthyretin; 2,4-dinitrophenol; amyloid fibrils; ANS; fluorescence quenching.**

Transthyretin (TTR),[2] a homotetrameric protein found in the plasma and cerebrospinal fluid (CSF), is involved in the transport of thyroxine and retinol. Normally TTR exists as an innocuous and soluble protein, but in some individuals TTR polymerizes to form amyloid fibrils (1–4). Amyloid fibril formation and deposition have been associated with various neurodegenerative disorders (2). TTR amyloidogenesis has been shown to be associated with familial amyloid polyneuropathy (FAP) and senile systemic amyloidosis (SSA). In FAP, mutated TTR was shown to be the causative factor and over 70 different amyloidogenic mutants of TTR have been identified. SSA, a wide spread geriatric disease, is associated with deposition of fibrils of wild-type TTR in the cardiac tissue (3, 4). However, at acidic pH both mutated and wild-type TTR were shown to form amyloid fibrils *in vitro*, suggesting low pH environment as the prerequisite for the amyloid formation, which is possible during endocytic pathway (5). Therefore, acid-induced fibril formation is being used as a model system to study the mechanistic aspects of amyloidogenesis (5). Recently Quintas *et al.* proposed a mechanism where TTR can dissociate into a nonnative monomer in solution at neutral pH, which will be in equilibrium with soluble aggregates, leading to fibril formation (6).

In most of the amyloid diseases, a conformational change in the native protein leads to the formation of amyloidogenic intermediate states, which can self-assemble into amyloid fibrils. Thus inhibition of the fibril formation is considered to be a potential strategy for therapeutic intervention (7–9). Previously TTR amyloid fibril formation was shown to be inhibited by T_4 and triiodophenol (TIP) via protein stabilization by ligand binding to the protein (7). In addition, small molecular weight, hydrophobic ligands like nitrophenols have been shown to block the amyloid β-aggregation *in vitro*. Neuroprotective effects of nitrophenols against amyloid β-induced neurotoxicity in rat hippocampal neurons have also been demonstrated (8). Weak hydrophobic interactions are considered to be

[1] To whom correspondence and reprint requests should be addressed. Fax: 91-40-7019074. E-mail: dr_sivakumarb@yahoo.com.

[2] Abbreviations used: TTR, Transthyretin; CSF, Cerebrospinal fluid; FAP, Familial amyloid polyneuropathy; SSA, Senile systemic amyloidosis; 2,4-DNP, 2,4-dinitrophenol; ANS, 8-Anilinonaphthalene-1-sulfonic acid; CHAPS, 3-[(3-chomidopropyl) dimethyl ammonio]-1 propanesulfonate; SDS–PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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responsible for the slow down of amyloid fibril formation at low temperatures and therefore, small molecular weight hydrophobic compounds could be used in destabilizing and disaggregating amyloid fibrils (8).

In the present report, we show that 2,4-dinitrophenol (2,4-DNP), a small water-soluble hydrophobic ligand, binds to TTR both at physiological and acidic pH and is capable of inhibiting amyloid fibril formation by stabilizing the tetramer at acidic pH.

MATERIALS AND METHODS

Materials

2,4-DNP, glutaraldehyde, sodiumborohydrate, Chaps, deoxy cholate, molecular weight markers and 8-anilinonaphthalene-1-sulphonic acid were obtained from Sigma Chemical Co.; Congo red was from BDH chemicals. Human TTR antiserum was obtained from Behring (Germany). Unless otherwise specified all the other chemicals were of reagent grade.

Purification of TTR

TTR was purified from pooled human plasma of normal individuals according to the previously described method (9) and characterized by SDS–PAGE and immunoblotting using human TTR antiserum. Protein concentration was determined by using $A^{1\%} = 14.1$ at 280 nm (9).

TTR Amyloid Fibril Formation and Inhibition by 2,4-DNP

Light scattering. To study the TTR amyloid fibril formation as a function of pH, stock solution (8 mg/ml in 50 mM phosphate buffer containing 0.15 M NaCl, pH 7.5 (PBS)) of TTR was added to 1 ml of 50 mM sodium acetate buffer, with varying pH from 5 to 3.6 containing 100 mM KCl to a final concentration of 4 μM. Wherever necessary, freshly made 2,4-DNP in water was added immediately to the TTR containing tubes to a final concentration of 5–20 μM and incubated at room temperature for different time periods up to 72 h. To asses the disaggregating effect of 2,4-DNP on preformed TTR amyloid fibrils, 2,4-DNP was added to a final concentration of 20 μM to the preformed fibrils and incubated for further 72 h. The extent of amyloid fibril formation was measured by monitoring the light scattering at 400 nm against respective blanks in a Hitachi (220S) spectrophotometer.

Congo red binding. Amyloid fibril formation was also assessed by Congo red, an amyloid fibril specific dye. TTR was incubated at desired pH as described above. At the end of 72 h incubation, 50 μl of TTR suspension was added to 1150 μl of 5 mM PBS containing 10 μM Congo red and incubated for further 1 h. The amount of Congo red bound was estimated as described previously, using the equation, moles of Congo red bound/L of amyloid suspension = $A_{540nm}/25295 - A_{477nm}/46306$ (10).

Fluorescence spectroscopy. All fluorescence measurements were performed at a controlled temperature of 25°C in a cuvette (1-cm lightpath) using a Perkin–Elmer Spectrofluorimeter (LS-3B). Intrinsic tryptophan fluorescence of TTR was followed in the absence and presence of 2,4-DNP at both acidic and physiological pH. TTR (0.27 μM) in either sodium acetate buffer, pH 4.4, or in the phosphate buffer, pH 7.5, was incubated for 24 h at room temperature with and without 5 μM 2,4-DNP. At the end of incubation, the emission spectrum (300–400 nm) was recorded by exciting at 280 nm. Both the excitation and emission slits were set to 10-nm band pass. To determine 2,4-DNP binding constants, small aliquots of 2.0 mM 2,4-DNP solution were successively added to 2.15 μM TTR in 50 mM phosphate buffer. After each addition of the ligand, the mixture was thoroughly mixed and left for 5 min (such an incubation period was found to be sufficient to establish equilibrium). Fluorescence intensity was then measured and the readings were corrected for buffer blanks and dilution. The calibration factor that relates the change in fluorescence intensity to the amount of 2,4-DNP bound to the protein was determined by a reverse titration method (11). Binding studies of TTR–ANS complex with 2,4-DNP were done by incubating 0.5 μM of TTR with 20 μM ANS. Fluorescence emission spectra (300–550 nm) were recorded by exciting at 280 nm. Fluorescence titration of TTR–ANS complex with 2,4-DNP was performed by adding small aliquots of concentrated 2,4-DNP. The concentration of TTR and ANS were 0.5 and 20 μM, respectively. Quenching of ANS fluorescence by 2,4-DNP was measured at 470 nm emission by exciting at 410 nm.

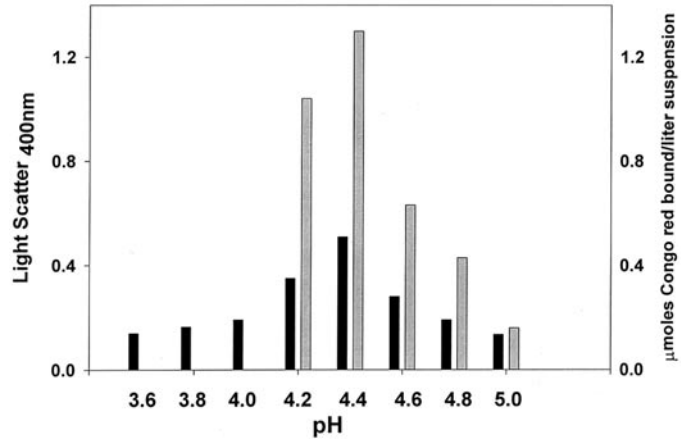

FIG. 1. TTR amyloid fibril formation as a function of pH. TTR (4 μM) was incubated at desired pH for 72 h and fibril formation was assessed by light scattering (black bars) and quantitative Congo red binding (shaded bars).

Glutaraldehyde cross-linking and SDS–Polyacrylamide gel electrophoresis. To monitor the quaternary structural changes of TTR at pH 4.4 in the presence and absence of 2,4-DNP, a SDS–PAGE method previously described was used (9). In the present method we have used Chaps as a detergent to prevent reassociation of protein during neutralization of pH before glutaraldehyde crosslinking. A 200-μl solution of TTR (0.4 mg/ml) was incubated at pH 4.4 in the presence and absence of 10 μM 2,4-DNP at 4°C for 24 h and then brought to 25°C for another 24 h, taking advantage of the earlier observation that TTR does not aggregate at low temperatures, there by allowing to monitor the structural changes (10). At the end of incubation, 10 μl of Chaps (60 mg/ml) and 70 μl of 0.5 M phosphate buffer, pH 7.5, were added to each sample for neutralization. Immediately, 200-μl aliquots were taken into fresh vials containing 6 μl of 25% glutaraldehyde. The cross-linking was allowed exactly for 3 min and further reaction was stopped by the addition of 10 μl of $NaBH_4$ (7 g/100 ml 0.1 M NaOH). To this, 800 μl of water, 8 μl of 12.5% deoxycholate, and 50 μl of 80% trichloroacetic acid were added to precipitate the protein. The precipitates were collected after centrifugation at 10,000 rpm for 20 min and washed twice with acetone and air-dried under nitrogen. The precipitates were mixed with 20 μl of 5% SDS-sample buffer and the boiled samples were loaded onto 12.5% SDS–PAGE. TTR incubated at pH 7.5 was used as a control and processed as described above except neutralization step. The gels were developed with Coomassie brilliant blue stain and analyzed by using Bio-Rad GS-710 imaging densitometer.

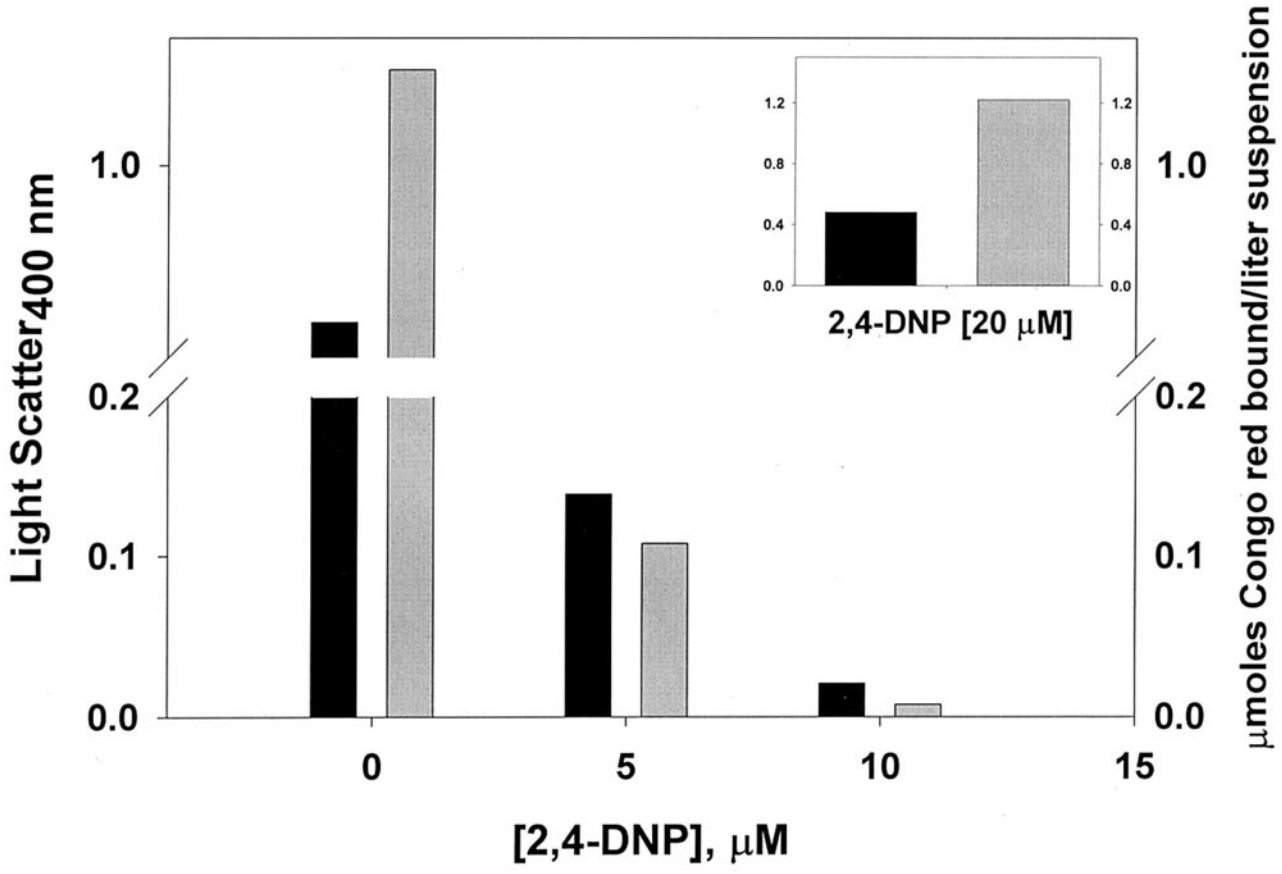

FIG. 2. Inhibition of TTR amyloid fibril formation by 2,4-DNP. TTR (4 μM) was incubated in the presence and absence of 2,4-DNP at pH 4.4 and the extent of fibril formation was monitored after 72 h by light scattering (black bars) and by quantitative Congo red binding (shaded bars). Inset: Preformed TTR fibrils incubated in the presence of 2,4-DNP.

RESULTS AND DISCUSSION

Preliminary studies indicated that light scattering due to fibril formation reached a plateau by 60–70 h (data not shown). Therefore, subsequent incubations were carried out for 72 h. As shown in Fig. 1, the fibril formation (ΔOD at 400 nm) was maximum at pH 4.4 as measured by both light scattering and Congo red binding. The Congo red-fibril suspension yielded a specific green birefringence when viewed under polarized microscope further confirming the amyloid nature of these aggregates (data not shown). However the extent of fibril formation could not be compared with Congo red binding below pH 4.2, as Congo red is known to precipitate at this pH (10). These results are in agreement with earlier reported data on TTR amyloid fibril formation, in which it was shown that at pH 4.4 relatively high amount of fibrils are formed as measured by light scattering and Congo red binding assay (10). Since, the fibril formation was maximum at pH 4.4 further studies were carried out at this pH.

Incubation of TTR along with 5–10 μM 2,4-DNP has not resulted in the fibril formation (Fig. 2). Though it has been demonstrated that 2,4-DNP disaggregates the preformed amyloid β-fibrils (8), it was ineffective in disaggregating preformed TTR fibrils (Fig. 2, inset). To understand further, the conformational states of TTR were followed by fluorescence spectroscopy in the presence and absence of 2,4-DNP. As shown in Fig. 3, TTR emission maximum was shifted to red (340 nm) at pH 4.4 and reduced in intensity compared to that of pH 7.5, where the emission maximum was at 336 nm, which suggests that TTR has undergone subtle conformational change at pH 4.4. Interestingly, in the presence of 2,4-DNP no red shift was observed at acidic pH implying that 2,4-DNP might have stabilized the protein. However, 2,4-DNP considerably and similarly quenched the TTR fluorescence at both pH 4.4 (62%) and pH 7.5 (58%), which implies that it may be interacting with the protein independent of pH. Therefore, we have investigated the binding of 2,4-DNP to TTR at pH 7.5. Figure 4 shows that 2,4-DNP indeed binds to TTR with a stiochiometric ratio of 6.5:1 and K_d of 6.4×10^{-8} M.

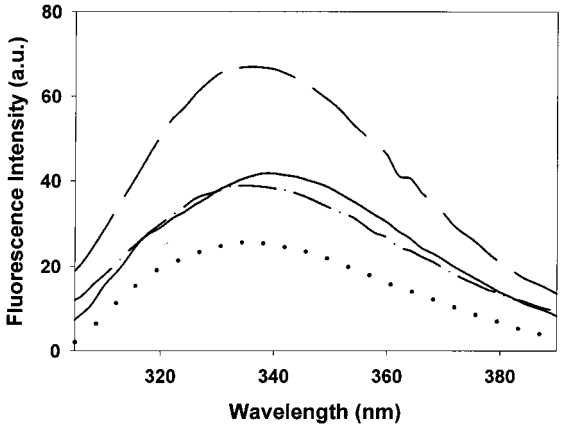

FIG. 3. Fluorescence emission spectra of TTR. TTR (0.27 μM) was incubated at pH 7.5 in the absence (dashed line) or in the presence of 2,4-DNP (dash-dot line) and at pH 4.4 in the absence (solid line) or in the presence of 2,4-DNP (dotted line). Low concentrations of TTR was used to avoid amyloid fibril formation at acidic pH and to follow conformational changes (10).

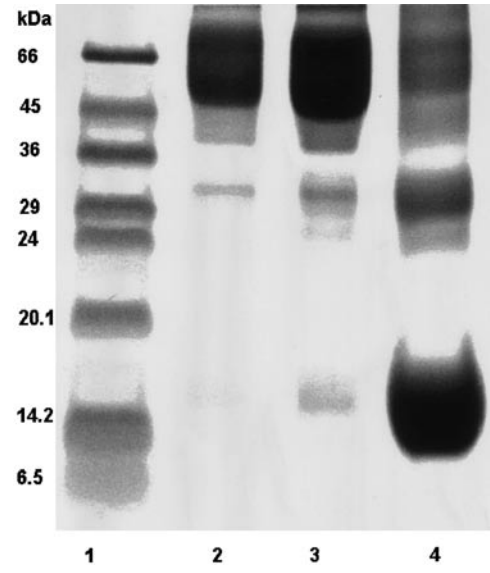

FIG. 5. SDS–polyacrylamide gel showing quaternary structure of TTR after cross-linking with glutaraldehyde. Lane 1, Molecular weight markers; Lane 2, TTR at pH 7.5; Lane 3, TTR +2,4-DNP at pH 4.4; Lane 4, TTR at pH 4.4.

Earlier studies suggested that TTR amyloid fibril formation involves the formation of amyloidogenic monomeric intermediate at acidic pH (5, 6). In addition, a strong correlation was found between the instability of the TTR tetramer and amyloidogenecity of the protein (12). These results together indicate that stability of the tetramer may be the key factor in the amyloidogenesis. Moreover, several small molecular weight ligands, which are shown to inhibit TTR amyloidogenesis, act via protein stabilization by binding to the active site of protein (7, 13, 14). The quaternary structure of TTR (tetramer or monomer) in the presence and absence of 2,4-DNP at acidic pH was characterized by glutaraldehyde cross-linking followed by SDS–PAGE. As shown in Fig. 5, TTR at pH 7.5 moved predominately as a tetramer, as discerned by a major band at 57 kDa with a relative quantity of 90%, where as at pH 4.4, most of the TTR moved as 14.5-kDa monomer (62%) with small quantities of both dimer (10%) and tetramer (28%). These results are in complete agreement with the earlier reported pH-dependent changes in TTR quaternary structure (9, 10). Interestingly, in the presence of 2,4-DNP, 85% of TTR remained as a tetramer with negligible quantities of monomer and dimer present at pH 4.4, suggesting that 2,4-DNP stabilizes TTR tetramer against pH-induced dissociation into monomeric, amyloid intermediate, and thereby inhibits amyloid fibril formation, observed through light-scattering and Congo red binding assays (Fig. 2). The mechanism of tetramer stabilization inturn could be due to inhibition of conformational changes in the TTR that are necessary for monomer formation (Fig. 3).

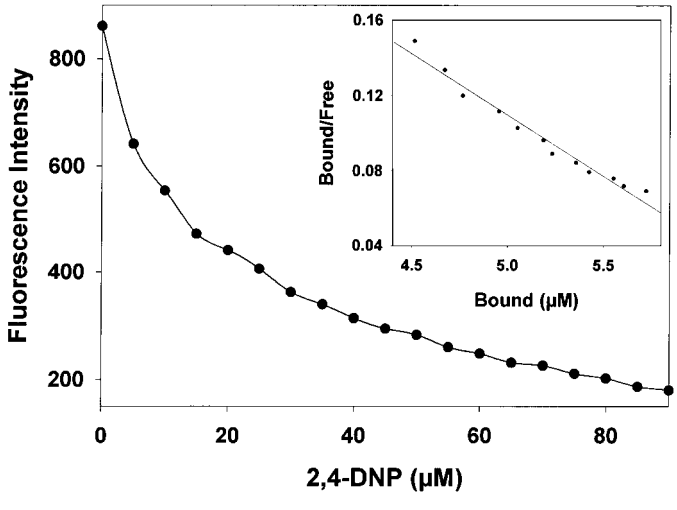

FIG. 4. Representative fluorescence titration of TTR with 2,4-DNP. TTR (2.15 μM) was titrated with small aliquots of 2.0 mM 2,4-DNP solution and the fluorescence intensity was monitored at 336 nm by exciting at 280 nm. Inset: Scatchard plots for the binding of 2,4-DNP to TTR. The concentration of protein bound 2,4-DNP was determined by reverse titration method as described earlier (11).

The results presented herein demonstrate that 2,4-DNP is an effective inhibitor of TTR amyloid fibril formation *in vitro* at concentrations of 5–10 μM. It is important to note that there exists a delicate balance between fibril formation and fibril clearance *in vivo* (15), and a compound that is moderately effective *in vitro* may prove to be more effective *in vivo*, because it will only be necessary to slightly shift the balance between formation and degradation of amyloid fibrils

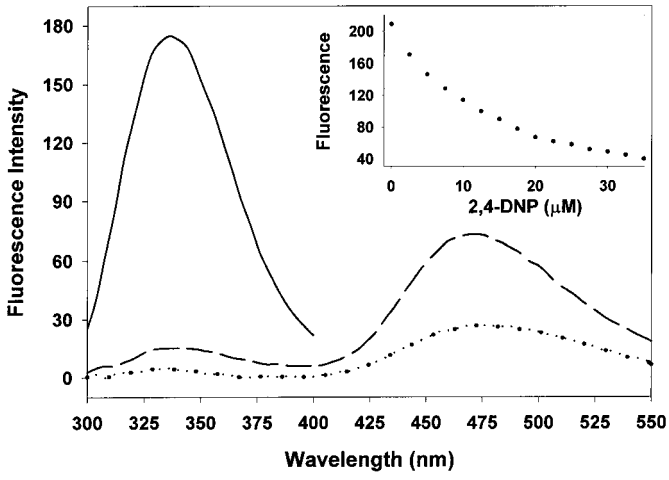

FIG. 6. Fluorescence emission spectra of TTR-ANS complex in presence and absence of 2,4-DNP. Fluorescence emission spectra of 0.5 μM TTR alone (solid line), in presence of 20 μM ANS (dashed line) and in the presence of both 20 μM ANS and 5 μM 2,4-DNP (dotted line). Inset: Fluorescence titration of ANS-TTR with 2,4-DNP. Concentration of TTR and ANS were 0.5 and 20 μM, respectively.

to avoid the onset of amyloid disease (16). Thus, much lower concentrations of 2,4-DNP might be sufficient *in vivo* to inhibit amyloidogenesis. The Scatchard analysis (Fig. 4) indicates 6.5 molecules of 2,4-DNP binding to one molecule of TTR. TTR is known to bind two molecules of T_4 (i.e., four phenyl rings) (7), logically TTR can accommodate four molecules of 2,4-DNP at the active site, suggesting that 2,4-DNP might be binding to the other sites on the protein in addition to the active site. We have used a fluorescent dye ANS as a probe to study the binding of 2,4-DNP to TTR active site. The fact that the 2 molecules of ANS bound to TTR can be displaced by T_4 suggests that ANS binds to the active site of TTR (17). As shown in Fig. 6, binding of ANS to TTR increases the ANS fluorescence by 10-fold and its emission maximum shifted to shorter wavelengths. On the other hand, ANS also quenched TTR fluorescence considerably as evidenced by the decrease in the fluorescence at 336 nm. Interestingly, addition of 2,4-DNP to TTR–ANS complex quenches the fluorescence of both ANS and protein, indicates that 2,4-DNP binding involves both active site as well as other sites on the protein. Furthermore, quenching of ANS fluorescence by 2,4-DNP follows saturation kinetics, supporting its binding to the active site (Fig. 6, inset). However, further characterization of binding sites involved in the interaction with 2,4-DNP, and its amyloid inhibitory potential against mutant forms of TTR is of great interest. But we should consider the fact that different small molecular weight compounds that inhibit TTR amyloidogenesis were shown to bind to the active site of the protein and did not show much difference in their capacity of amyloid inhibition whether or not there was a mutation in TTR (7, 13, 14). This could mean that T_4 binding site of TTR was unaltered in most of the point mutations.

In conclusion, these *in vitro* studies demonstrate that 2,4-DNP binds to TTR at active site as well as at other sites and inhibits amyloid fibril formation through tetramer stabilization at pH 4.4. These observations may help us in designing small molecular weight hydrophobic compounds using nitrophenols as lead compounds, which may have implications in the treatment of TTR amyloid diseases. Since 2,4-DNP at submicromolar concentrations is effective in inhibiting TTR fibril formation *in vitro*, the efficacy of this compound against TTR amyloidogensis *in vivo* remains to be explored.

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