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Effect of NBD chloride (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) on the pyridine nucleotide transhydrogenase of *Escherichia coli*

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Abstract

Pyridine nucleotide transhydrogenases of bacterial cytosolic membranes and mitochondrial inner membranes are proton pumps in which hydride transfer between $NADP^+$ and NAD^+ is coupled to proton translocation across cytosolic or mitochondrial membranes. The pyridine nucleotide transhydrogenase of *Escherichia coli* is composed of two subunits (α and β). Three domains are recognized. The extrinsic cytosolic domain 1 of the amino-terminal region of the α subunit bears the NAD(H)-binding site. The NADP(H)-binding site is present in domain 3, the extrinsic cytosolic carboxyl-terminal region of the β subunit. Domain 2 is composed of the membrane-intrinsic carboxyl-terminal region of the α subunit and the membrane-intrinsic amino-terminal region of the β subunit. Treatment of the transhydrogenase of *E. coli* with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) inhibited enzyme activity. Analysis of inhibition revealed that several sites on the enzyme were involved. NBD chloride modified two (βCys-147 and βCys-260) of the seven cysteine residues present in the transhydrogenase. Modification of βCys-260 in domain 2 resulted in inhibition of enzyme activity. Modification of residues other than cysteine residues also resulted in inhibition of transhydrogenation as shown by use of a cysteine-free mutant enzyme. The β subunit was modified by NBD chloride to a greater extent than the α subunit. Reaction of domain 2 and domain 3 was prevented by NADPH. Modification of domain 3 is probably not associated with inhibition of enzyme activity. Modification of domain 2 of the β subunit resulted in a decreased binding affinity for NADPH at its binding site in domain 3. The product resulting from the reaction of NBD chloride with NADPH was a very effective inhibitor of transhydrogenation. In experiments with NBD chloride in the presence of NADPH it is likely that all of the sites of reaction described above will contribute to the inhibition observed. The NBD-NADPH adduct will likely be more useful than NBD chloride in investigations of the pyridine nucleotide transhydrogenase.

Keywords: Transhydrogenase; Pyridine nucleotide transhydrogenase; NBD chloride; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; Enzyme inhibitor; Cysteine residue

Abbreviations: $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MES, 2-(*N*-morpholino)ethane sulfonic acid; NBD chloride, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NBD-NADPH, product formed by the reaction of NBD chloride with NADPH; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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1. Introduction

Pyridine nucleotide transhydrogenase is a proton pump which catalyzes the reversible reaction

$H^+_{out}+NADP^+ + NADH \leftrightarrows NADPH + NAD^+ + H^+_{in}$,

where reduction of $NADP^+$ by NADH is linked to an inward translocation of protons from the periplasm (in bacteria) or cytosol (in mammalian cells) into the cytosol or mitochondrial matrix, respectively. The properties of transhydrogenases have been reviewed recently [1–4].

The enzyme in *Escherichia coli* is composed of two different subunits (α, 510 residues; β, 462 residues) assembled as an $\alpha_2\beta_2$ tetramer [5–8]. All membrane transhydrogenases are organized into three domains [1,9,10]. Domain 1 is a cytosolic domain which contains the binding site for NAD(H) and is the amino-terminal region of the α subunit. Domain 3 is a cytosolic domain found at the carboxyl-terminal region of the β subunit. It contains the NADP(H)-binding site. Domain 2 is the transmembranous region of the enzyme. It consists in *E. coli* of two parts: the carboxyl-terminal region of the α subunit, which terminates in four transmembrane α-helices, and the eight or nine transmembrane α-helices which compose the amino-terminal half of the β subunit. Proton translocation across domain 2 is coupled to hydride ion transfer between domains 1 and 3. The driving force for proton translocation is likely the difference in binding energies between the substrates and products of the reaction [1,11,12].

The reagent 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) has been used as an enzyme inhibitor. Its use has been particularly informative in studies of the mechanism of the F1-adenosine triphosphatase [13–18]. NBD chloride reacts at pH 7.0 with thiols and with the phenolic hydroxyl group of tyrosine. At higher pH values (8.0 and above) it will react with amino groups. The NBD group can also be transferred from tyrosine to lysine residues at the higher pH [13–16,19]. Persson et al. [20] have investigated the action of NBD chloride on the beef-heart mitochondrial transhydrogenase. They found that 4–6 cysteine residues of the 10 present in the enzyme reacted with the inhibitor. Of these, 1 or 2 cysteine residues were classified as 'fast-reacting' and 3 or 4 cysteine residues as 'slow-reacting'. All substrates of the transhydrogenase accelerated the rate of reaction of NBD chloride with the enzyme. Modification of the enzyme with NBD chloride resulted in inhibition, with the extent of inhibition being proportional to the degree of reaction of the inhibitor with the enzyme.

With the expectation that NBD chloride might provide some understanding of the role of sulfhydryl groups in transhydrogenation, we have investigated the reaction of NBD chloride with the pyridine nucleotide transhydrogenase of *E. coli*. Several sites of interaction of the inhibitor with the enzyme were identified. Inhibition of non-mutant transhydrogenase was accompanied by modification of the two cysteine residues of domain 2 of the β subunit. Cysteine-free mutant transhydrogenase was inhibited by NBD chloride, indicating that residues other than cysteine residues were modified by the inhibitor. The residues were probably lysine residues on domains 2 and 3 of the β subunit. The product derived from the reaction of NBD chloride with NADPH was an effective inhibitor of transhydrogenase activity. Thus, NBD chloride is not useful as an inhibitor of specific sites on the transhydrogenase. The NBD-NADPH adduct may be more useful for this purpose.

2. Materials and methods

2.1. Bacterial strain, plasmids and mutagenesis

E. coli JM109 cells containing wild-type (pSA2) or mutant plasmids were grown overnight at 37°C in LB broth. The medium was shaken at 250 rpm in a New Brunswick Scientific Controlled Environment incubator shaker. Plasmid pSA2 contains the pnt genes of the pyridine nucleotide transhydrogenase of *E. coli* introduced into the pGEM-7Zf(+) plasmid [7].

Plasmid pSA2 was used to isolate single-stranded phagemid DNA. Site-directed mutagenesis to convert selected residues was performed by the method of Taylor et al. [21] using degenerate primers. The reagents and protocols as outlined in the Amersham Sculptor and the Bio-Rad Muta-Gene phagemid in vitro mutagenesis kits were followed, except that competent *E. coli* JM109 cells were used for transformation. Plasmid DNA was prepared from individ-

ual colonies and the mutants were identified by double-stranded DNA sequencing. The mutated region was subcloned into non-mutated DNA and the mutated region sequenced.

Plasmid pCH25, coding for a cysteine-free transhydrogenase, was isolated as described in [22,23]. Plasmids pCH36 and pCH37 code for a cysteine-free transhydrogenase in which the codons for βCys-147 and βCys-260, respectively, have been restored.

2.2. Preparation of Triton-washed inner membrane vesicles containing transhydrogenase

The cell cultures were harvested by centrifugation at 4400×*g* for 20 min. The cell pellets were washed by resuspension in 0.9% NaCl followed by centrifugation at 12 000×*g* for 15 min. Cell pellets were resuspended in dithiothreitol (DTT)-free buffer A (50 mM Tris-HCl, pH 7.8, 1 mM EDTA) [24] at 1 g wet weight/5 ml. All steps were performed at 0–4°C. The cells were lysed by passage through an Aminco French pressure cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12 000×*g* for 10 min. The supernatant was centrifuged at 252 000×*g* for 2 h and the membrane pellet containing everted membrane vesicles was suspended in DTT-free buffer A at 1 g wet weight/5 ml. Membrane vesicles (1.5 ml) were layered on a 6 ml sucrose cushion (45% sucrose (w/w) in buffer A) and centrifuged in a Beckman Type 65 fixed angle rotor at 40 000 rpm (139 000×*g*) for 1 h. The outer membrane fraction pelleted to the bottom of the tube and was discarded while the everted inner membrane vesicles banded at the interface and were removed by a syringe. The vesicles were diluted with DTT-free buffer containing 1% (w/v) Triton X-100 and then centrifuged at 252 000×*g* for 3 h. The washed membrane pellet was suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)-KOH, pH 8, 5 mM magnesium acetate for use.

2.3. Purification of the transhydrogenases of pSA2 and pCH25

The crude membrane pellets, containing both inner and outer membranes, from 10–12 g cells were prepared as described in Section 2.2. The pellets were suspended in buffer B (50 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, 1 mM EDTA, 1 mg/ml Brij-35) and the transhydrogenases solubilized by stirring for 10 min with dodecyl maltoside at a concentration of 12.5 mg/ml. The mixture was centrifuged for 2.5 h at 252 000×*g*. The supernatant was applied to a DEAE-BioGel A column (17×2.5 cm) equilibrated with buffer B. A linear gradient of 0–0.25 M NaCl in buffer B (400 ml total volume) was applied. Fractions containing the transhydrogenase were applied to a column (115×2.5 cm) of Sepharose S-300 equilibrated with buffer B. Active fractions eluted from this column were stored individually at −65°C.

2.4. Measurement of transhydrogenation activities

Transhydrogenation of 3-acetylpyridine adenine dinucleotide (AcPyAD$^+$) by NADPH ('reverse transhydrogenation') was measured as described previously [24]. An appropriate amount of purified enzyme or membrane was added to 1 ml of 50 mM sodium phosphate buffer (pH 7), 0.5 mM EDTA, 2 mM DTT, 0.01% Brij-35 containing AcPyAD$^+$ and NADPH at 0.5 mM. Reduction of AcPyAD$^+$ was followed at 375 nm using a Perkin-Elmer Lambda 3A UV/VIS spectrophotometer.

Transhydrogenation of AcPyAD$^+$ by NADH in the presence of NADP$^+$ ('cyclic transhydrogenation') was measured at pH 6.0 in a buffer containing 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES)-KOH, 0.5 mM EDTA, and 0.01% Brij-35. AcPyAD$^+$ and NADH were present at a concentration of 0.5 mM. The concentration of NADP$^+$ was 0.1 mM.

Protein concentrations were determined by the method of Lowry et al. [25] or by using the BCA (bicinchoninic acid) protein assay kit supplied by Pierce (Rockford, IL, USA).

2.5. Reaction with NBD chloride

For most experiments purified transhydrogenases from pSA2 or pCH25, or Triton X-100 washed inner membrane vesicles, were incubated in the dark in 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 0.01% Brij-35, with NBD chloride. For cyclic transhydrogenation the enzymes or vesicles were incubated in the dark in 50 mM

MES-NaOH buffer, pH 6.0, containing 0.5 mM EDTA and 0.01% Brij-35, with NBD chloride.

The NBD-NADPH adduct was prepared by incubating 0.4 μmol NBD chloride with 0.5 μmol NADPH in 1 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 0.01% Brij-35, for 24 h in the dark. No free NBD chloride could be detected at this time and the absorption at 490 nm, indicative of amino group substitution by the inhibitor, was at its maximum value. The maximum contamination by free NADPH would be 20%.

2.6. Measurement of enzyme-bound NADPH

This measurement was carried out as described in [26]

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was carried out by the Laemmli method [27]. Gels were photographed under ultraviolet light before staining with Coomassie blue.

2.8. Materials

All biochemicals including coenzymes and NBD chloride were supplied by Sigma. DEAE BioGel A and polyacrylamide solutions were obtained from Bio-Rad. Sepharose S-300 and Sephadex G-50 were purchased from Pharmacia. LB broth was supplied by Difco.

3. Results

Most of the experiments in this study were carried out using purified pyridine nucleotide transhydrogenases encoded by plasmids pSA2 [7] and pCH25 [22,23]. The transhydrogenase from pSA2 is the normal, non-mutant enzyme whereas that from pCH25 lacks the seven cysteine residues present in the normal enzyme. The mutations are αC292T, αC339T, αC395S, αC397T, αC435S, βC147S and βC260S. In some experiments inner membrane vesicles which had been washed with a buffer containing Triton X-100 were used. The transhydrogenase composes at least 90% of the protein present in the vesicles.

3.1. Effect of NBD chloride on pyridine nucleotide transhydrogenase activity

NBD chloride inhibited the reduction of $AcPyAD^+$ by NADPH ('reverse transhydrogenation') by purified transhydrogenases from pSA2

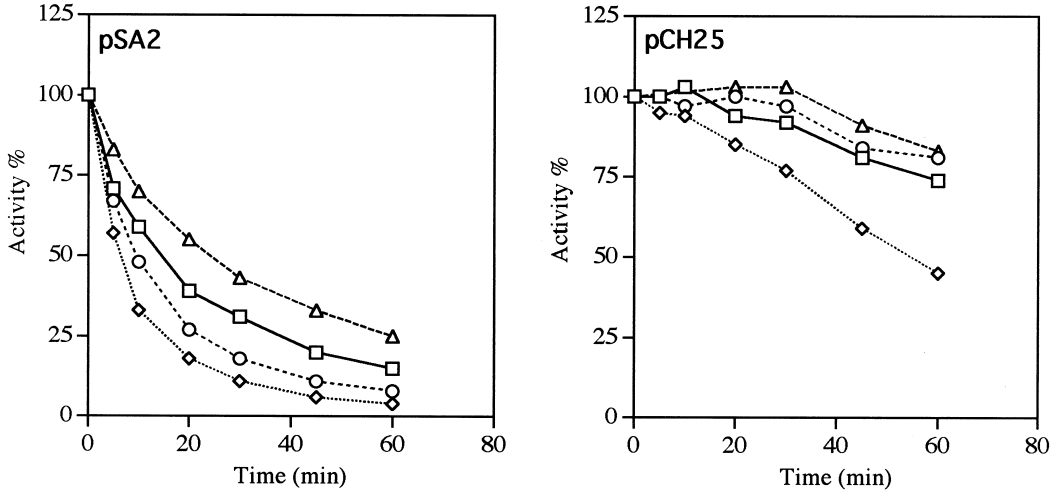

Fig. 1. Inhibition of reverse transhydrogenase activities of purified enzymes from pSA2 and pCH25 by NBD chloride in the absence and presence of pyridine nucleotide substrates. Transhydrogenase (pSA2, 0.15 mg protein/ml; pCH25, 0.37 mg protein/ml) was incubated in the dark with 0.4 mM NBD chloride in the absence of pyridine nucleotides (□), or with 0.5 mM NADPH (◇), $NADP^+$ (○) or NAD^+ (△) present. Reverse transhydrogenase activity was assayed at the indicated time points. Activity is expressed as a percentage of the initial activity.

and pCH25 (Fig. 1). The cysteine-free enzyme from pCH25 was less sensitive to inhibition. Substrates of the enzyme affected the rate of inhibition. NADPH enhanced the rate of inhibition with both strains. NAD^+ provided some protection against the inhibition. Similar results were obtained using Triton X-100 washed inner membrane vesicles. Addition of DTT to the inhibited enzymes did not reverse inhibition.

The concentration dependency of inhibition of reverse and cyclic transhydrogenation in the presence and absence of NADPH is shown in Fig. 2. In cyclic transhydrogenation hydride equivalents from NADH at the NAD(H) site on domain 1 are transferred via bound $NADP^+$ at the NADP(H) site of domain 3 to $AcPyAD^+$ now occupying the NAD(H) site. This reaction is considered to measure hydride transfer between the NAD(H) and NADP(H) sites in the absence of coupled proton translocation [28,29]. The transhydrogenase from pCH25 was significantly less sensitive than the enzyme from pSA2 to inhibition by NBD chloride in both reverse and cyclic transhydrogenation. In the presence of NADPH, which increased the sensitivity of the enzymes to inhibition,

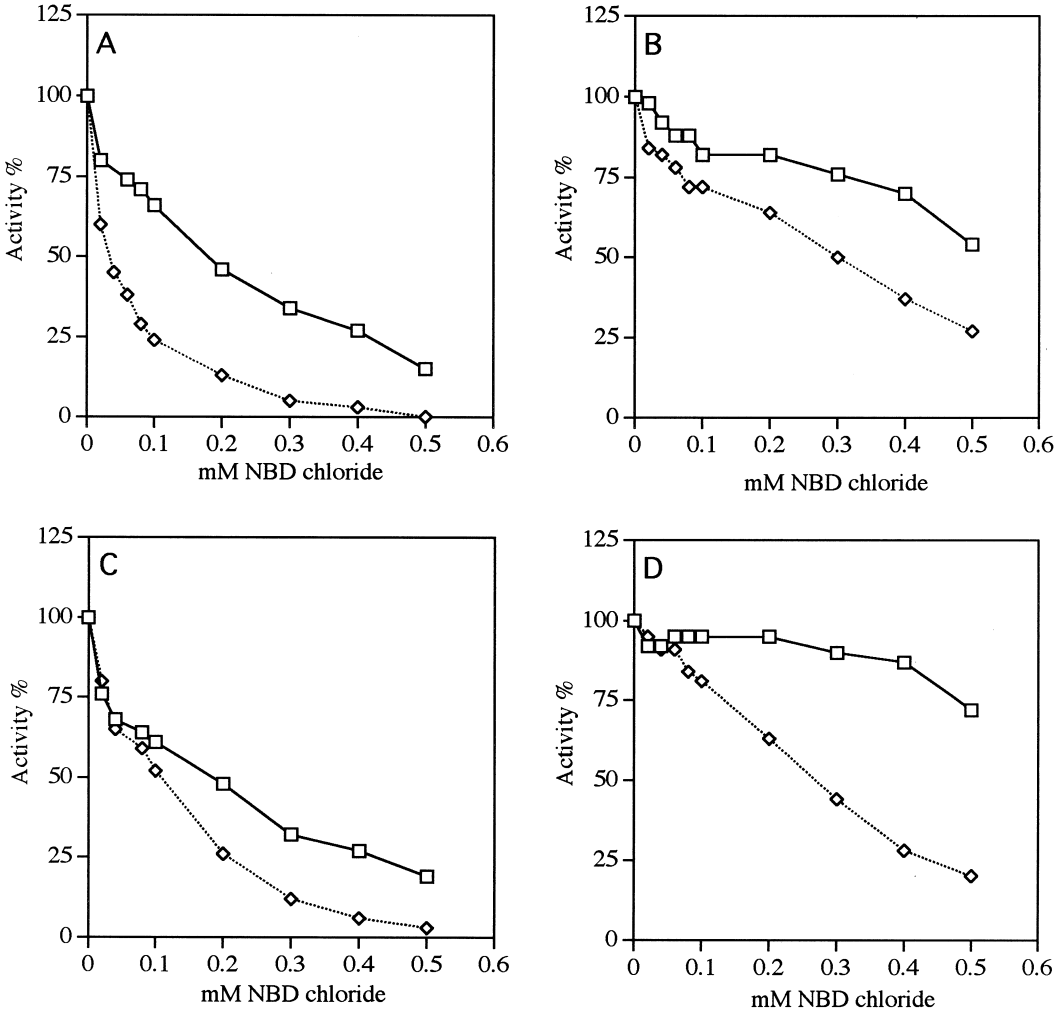

Fig. 2. Effect of concentration of NBD chloride on the inhibition of transhydrogenase activities in the absence or presence of 0.5 mM NADPH. Purified transhydrogenases (A, 0.76 mg protein/ml; B, 0.83 mg protein/ml; C, 0.70 mg protein/ml; D, 0.83 mg protein/ml) in the absence (□) or presence of 0.5 mM NADPH (◇) were incubated with the indicated concentration of NBD chloride in the dark for 2 h. The reaction was stopped by the addition of cysteine hydrochloride to a final concentration of 5.5 mM. Samples were analyzed for reverse (A and B) and cyclic (C and D) transhydrogenation. (A and C) pSA2; (B and D) pCH25. Activity is expressed as a percentage of the activity in the absence of inhibitor.

reverse transhydrogenation was inhibited 50% by 0.03 mM NBD chloride with the purified enzyme from pSA2 compared with 0.3 mM for the transhydrogenase from pCH25.

3.2. Spectrophotometric examination of the reaction of NBD chloride with pyridine nucleotide transhydrogenases

Comparison of the inhibition of transhydrogenation in the non-mutant and cysteine-free enzymes of pSA2 and pCH25, respectively, suggested that the increased inhibition of transhydrogenation in pSA2 was due to modification of cysteine residues. However, the sensitivity of the enzyme from pCH25 to inhibition by NBD chloride clearly indicated that other residues must be involved in inhibition. Purified transhydrogenases from pSA2 and pCH25 reacted with NBD chloride in the presence of NADPH. The absorption spectra of the modified enzymes were measured after removal of excess inhibitor on centrifuged columns of Sephadex G-50. The modified enzyme from pSA2 had an absorption band at 425 nm (Fig. 3). This band is characteristic

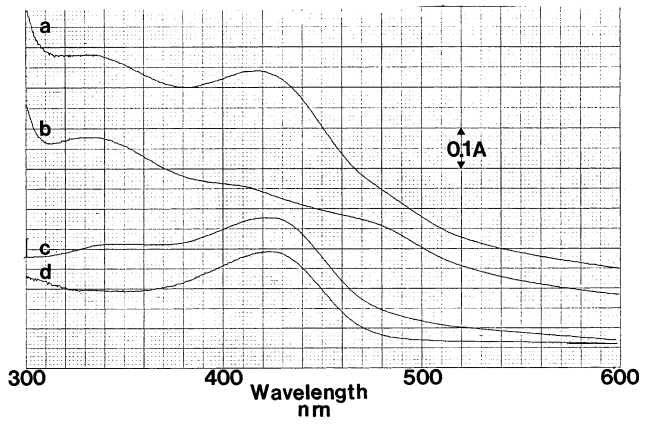

Fig. 3. Absorption spectra of purified transhydrogenases from pSA2 and pCH25 treated with NBD chloride. The enzymes (0.66 mg protein) in 1 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA, 0.01% Brij-35 and 0.5 mM NADPH were treated with 0.5 mM NBD chloride in the dark for 2 h. The samples were then passed through centrifuged 10 ml columns of Sephadex G-50 equilibrated with the same buffer but without NADPH. Absorption spectra of the treated enzymes were measured in a SLM-Amino DW-2C spectrophotometer. Spectrum a, pSA2; b, pCH25; c, measured difference spectrum between pSA2 and pCH25; d, calculated difference spectrum between pSA2 and pCH25.

of the reaction of NBD chloride with the sulfhydryl group of cysteine residues [13]. This band was not visible in pCH25 and was most clearly seen in the difference spectrum between the inhibitor-modified enzymes from pSA2 and pCH25 (Fig. 3). The absorption band at 425 nm was used to calculate the extent of modification of the seven cysteine residues which are present in the transhydrogenase from pSA2. Using the extinction coefficient determined by Persson et al [20], a value of 2.15 ± 0.02 modified cysteine residues/$\alpha\beta$ molecule was obtained.

Both of the modified enzymes from pSA2 and pCH25 showed small absorption bands at 340 and 480 nm. The latter band was due to the reaction of NBD chloride with amino groups [13,19]. Since NADPH was present during reaction of NBD chloride with the inhibitor, the possibility that the 340 nm band was due to the presence of this pyridine nucleotide bound to the enzyme was examined. Nucleotide analysis [26] showed that NADPH was absent.

3.3. Location of the modified groups in the transhydrogenases from pSA2 and pCH25

Purified transhydrogenases from pSA2 and pCH25 were treated with NBD chloride in the presence and absence of NADPH and then examined by electrophoresis on SDS-polyacrylamide gels. The presence of bound NBD was shown by fluorescence under ultraviolet light. As shown in Fig. 4A, the β subunit was modified to a greater extent than the α subunit in the enzymes of both strains. The presence of NADPH during treatment with NBD chloride reduced the extent of modification of the β subunit such that both α and β subunits were labeled to a similar extent.

Trypsin in the presence of NADPH cleaves the α and β subunits to yield well-recognized fragments [30]. The α subunit is cleaved by trypsin to yield initially a 43 kDa moiety which is essentially domain 1. This moiety is subsequently cleaved to give 29 and 16 kDa fragments. The β subunit is cleaved in the presence of NADPH to yield 30 and 25 kDa fragments. The 30 kDa fragment is essentially domain 3. The 25 kDa fragment originates from the amino-terminus of the β subunit and is part of domain 2. The NBD-modified transhydrogenases from pSA2 and pCH25 were treated with trypsin in the presence of

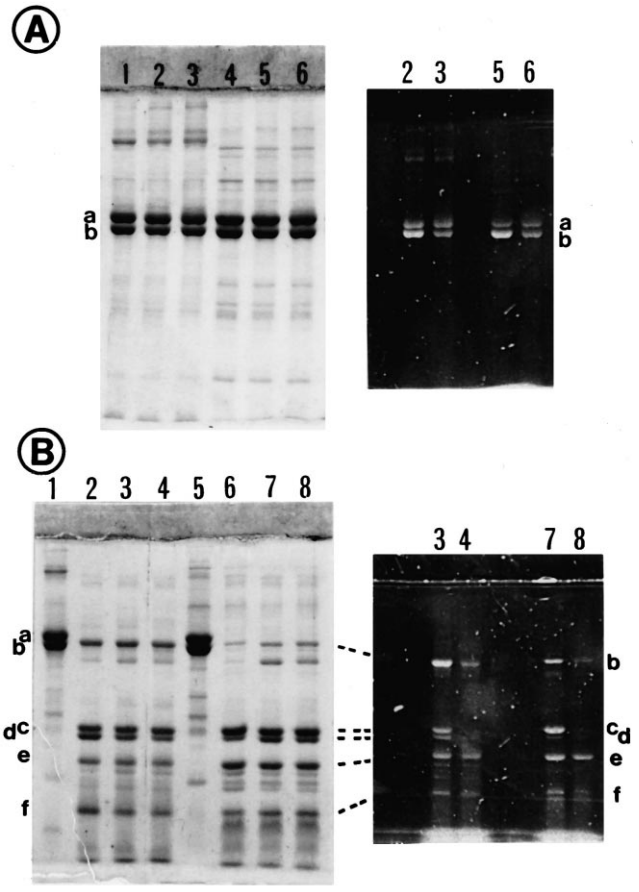

Fig. 4. SDS-PAGE of NBD chloride-treated purified transhydrogenases from pSA2 and pCH25. The gels on the left-hand side were stained with Coomassie blue. On the right-hand side are photographs of the same gels taken under ultraviolet light before staining. (A) 1, untreated enzyme from pSA2; 2 and 3, NBD chloride-treated enzyme from pSA2 treated in the absence or presence of NADPH, respectively; 4, like 1, but with enzyme from pCH25; 5 and 6, like 2 and 3 but with enzyme from pCH25. (B) 1, untreated enzyme from pSA2; 2, untreated enzyme from pSA2 digested with trypsin in the presence of NADPH; 3 and 4, enzyme from pSA2 treated with NBD chloride in the absence or presence of NADPH, respectively, and then digested with trypsin in the presence of NADPH; 5, like 1, but with enzyme from pCH25; 6, like 2, but with enzyme from pCH25; 7 and 8, like 3 and 4, but with enzyme from pCH25. The enzymes were treated with 0.4 mM NBD chloride in the dark for 1.5 h (A) and 2 h (B). The concentration of acrylamide was 10% (w/v)(A) and 11% (w/v)(B). Trypsin digestion was carried out as described in [30]. a and b denote α and β subunits of the transhydrogenase. c and e denote the 30 and 25 kDa tryptic fragments of the β subunit. d and f denote 29 and 16 kDa tryptic fragments of the α subunit.

NADPH (Fig. 4B). The 30 and 25 kDa fragments of the β subunit were the most strongly labeled polypeptides seen under ultraviolet illumination. The 29 and 16 kDa polypeptides from the α subunit were labeled to a lesser degree. Residual undigested β subunit was also strongly fluorescent. The presence of NADPH during labeling dramatically lowered the labeling of the 30 kDa polypeptide. Since this polypeptide is essentially domain 3, which carries the binding site for NADP(H), it is likely that NBD chloride labeled a site close to this binding site and that substrate protected against labeling. The enzymes from pSA2 and pCH25 behaved similarly in these experiments, indicating that labeling of residues other than cysteine residues had occurred. These are most likely amino groups since NBD chloride yields fluorescent products only with sulfhydryl and amino groups. The phenolic hydroxyl group of tyrosine does not yield a fluorescent product [13].

3.4. Location of modified cysteine residues in the transhydrogenase from pSA2

The non-mutant transhydrogenase from pSA2 contains seven cysteine residues, five on the α subunit and two on the β subunit [7,22,23]. The experi-

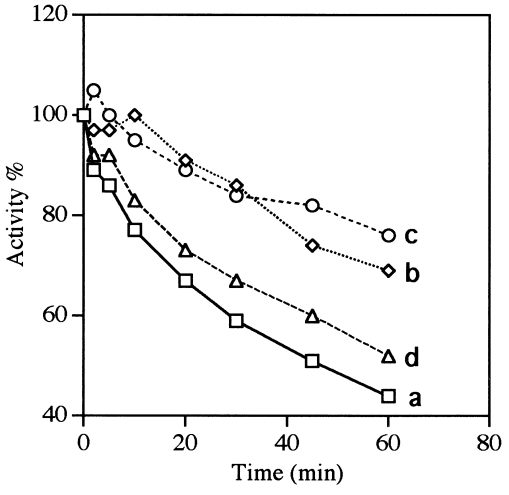

Fig. 5. Effect of NBD chloride on the reverse transhydrogenase activities of Triton X-100 washed inner membrane vesicles from pSA2 (curve a), pCH25 (curve b), pCH36 (curve c) and pCH37 (curve d). Membrane vesicles (0.43 mg protein/ml) were incubated in the dark with 0.4 mM NBD chloride. Samples were assayed for reverse transhydrogenase activity at timed intervals. Activity is expressed as a percentage of the initial activity.

ments described in the previous section (Section 3.3) suggested that, in the presence of NADPH, the major site(s) of modification by NBD chloride was in the amino-terminal 25 kDa region of the β subunit. This portion of domain 2 contains two cysteine residues, βCys-147 and βCys-260. Triton X-100 washed inner membrane vesicles were used to examine the role of these residues in the sensitivity of the transhydrogenase to inhibition by NBD chloride. Two mutants were used. The transhydrogenase from pCH36 contained only βCys-147, the remaining cysteines of the enzyme having been mutated to serine or threonine residues. The enzyme from pCH37 was similar except that βCys-260 was the only cysteine residue. As shown in Fig. 5, the transhydrogenase from pCH36 showed a sensitivity to NBD chloride similar to that of pCH25 (cysteine-free), whereas the enzyme from pCH37 more closely resembled the enzyme from pSA2 (non-mutant). Examination of the absorption spectra of NBD chloride-treated Triton X-100 washed inner membrane vesicles from pCH36 and pCH37 revealed the band at 425 nM characteristic of the product of the reaction of NBD chloride with cysteine residues. Calculations from the intensity of this band showed 0.98 and 0.57 mol cysteine/mol αβ had been modified in the transhydrogenases of pCH36 and pCH37, respectively.

The above results indicate that modification of βCys-260 by NBD chloride was responsible for the inhibition of transhydrogenase activity.

3.5. *Effect of other mutations on inhibition of transhydrogenase activity*

The effect of NBD chloride on reverse transhydrogenation with Triton X-100 washed inner membrane vesicles of various mutants was studied. Since tyrosine residues are potential targets for the inhibitor, we examined the enzymes with mutations αY226F/βY315F/βY431F (conserved tyrosine residues in the NAD(H) and NADP(H)-binding sites), or αY439F and βY224F (the conserved tyrosine residues of domain 2). We also examined transhydrogenases containing the conserved lysine residues αK228T (NAD(H)-binding site) or βK145T (the only conserved lysine residue of domain 2). Finally, as the residues βHis-91, βAsp-213 and βAsn-222 have been implicated in transmembrane proton pumping by the transhydrogenase [31–33], we examined transhydrogenases containing the mutations βH91N, βD213N or βN222D.

None of the mutations described above provided resistance to inhibition of reverse transhydrogenation by NBD chloride (data not shown).

3.6. *Effect of NBD chloride on the kinetics of reduction of AcPyAD$^+$ by NADPH (reverse transhydrogenation)*

Incubation of purified transhydrogenase or Triton X-100 washed membrane vesicles with NBD chloride in the presence of NADPH resulted in a lag in the reduction of AcPyAD$^+$ by NADPH before the final

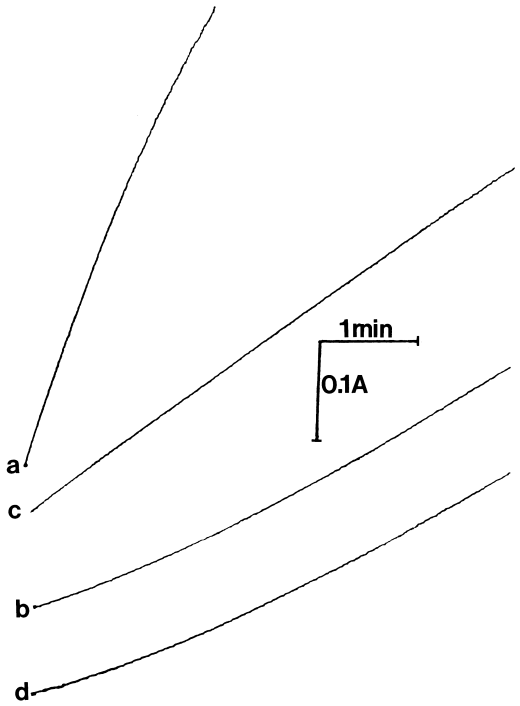

Fig. 6. Effect of preincubation of NBD chloride-treated Triton X-100 washed inner membrane vesicles from pCH25 with AcPyAD$^+$ or NADPH on the lag in the reverse transhydrogenase assay. Vesicles (0.64 mg protein/ml) were treated with 0.4 mM NBD chloride in the presence of 0.5 mM NADPH in the dark for 75 min and then assayed for reverse transhydrogenation. a, control - no inhibitor; b, NBD-treated; c, NBD-treated, then preincubated with 0.5 mM NADPH for 5 min before the assay of reverse transhydrogenation was started; d, same as c but with the modified enzyme preincubated with 0.5 mM AcPyAD$^+$. The change in absorption was measured at 375 nm.

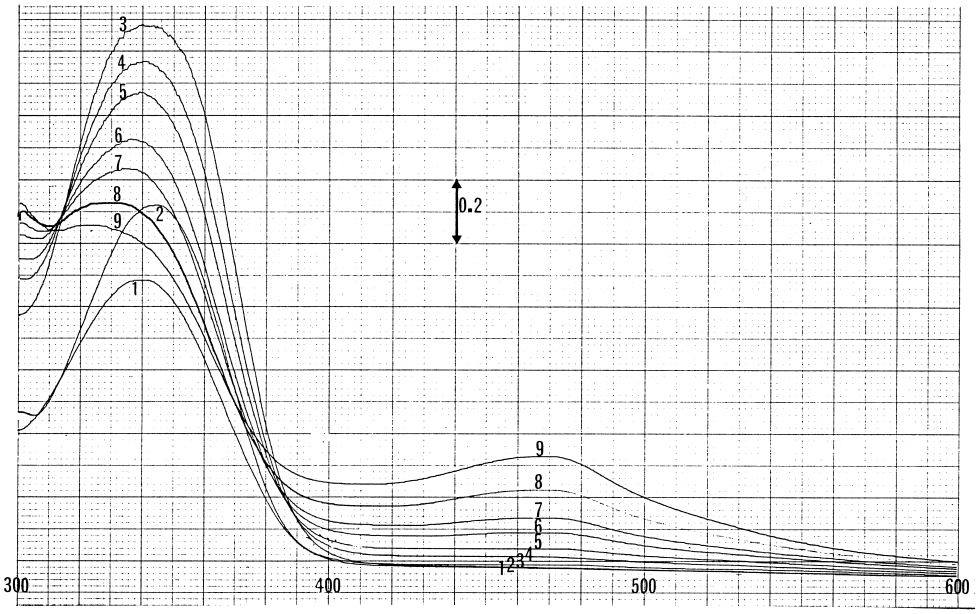

Fig. 7. Changes in the absorption spectrum with time of a mixture of NADPH and NBD chloride. NBD chloride (0.4 μmol) and NADPH (0.5 μmol) were incubated in the dark in sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 0.01% Brij-35. The absorption spectrum was measured at timed intervals in a SLM-Aminco DW-2C spectrophotometer. 1, NADPH alone; 2, NBD chloride alone; 3–9, spectra of the mixture at 0, 0.5, 1, 2, 3, 5 and 7 h.

rate was attained. This is illustrated in Fig. 6 for X-100 washed inner membrane vesicles from pCH25. Preincubation of the inhibited enzyme with 0.5 mM NADPH for 5 min before the assay was initiated by addition of $AcPyAD^+$ abolished the lag period. Preincubation with 0.5 mM $AcPyAD^+$ had no effect. It is concluded that NBD chloride modification in the

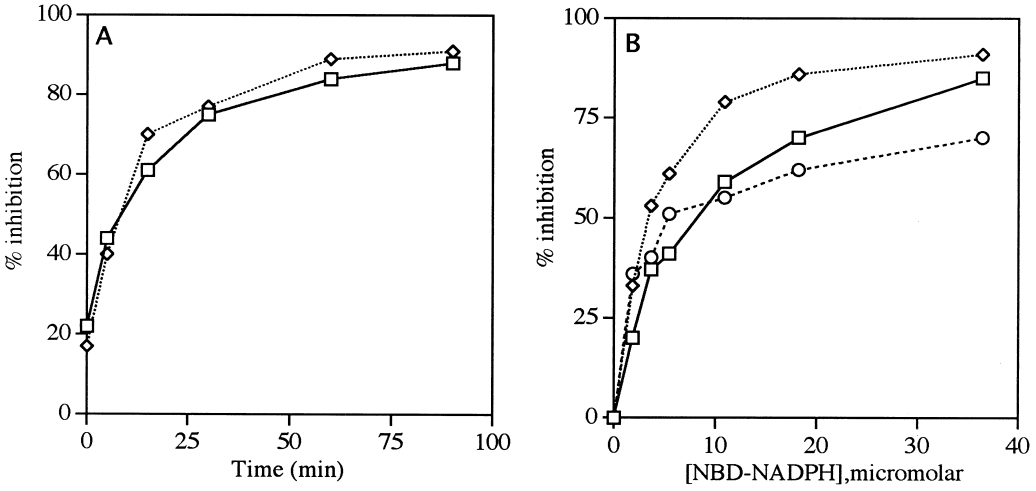

Fig. 8. Inhibition by the NBD-NADPH adduct of the transhydrogenase activities of purified enzymes from pSA2 and pCH25. (A) The transhydrogenases from pSA2 (□) and pCH25 (◇) were preincubated in the dark for the indicated time with 18.2 μM NBD-NADPH adduct before assay for reverse transhydrogenase activity. (B) The transhydrogenases from pSA2 (□,○) and pCH25 (◇) were preincubated in the dark for 30 min with the indicated concentration of the NBD-NADPH adduct before assay for reverse (□, ◇) and cyclic (○) transhydrogenase activities.

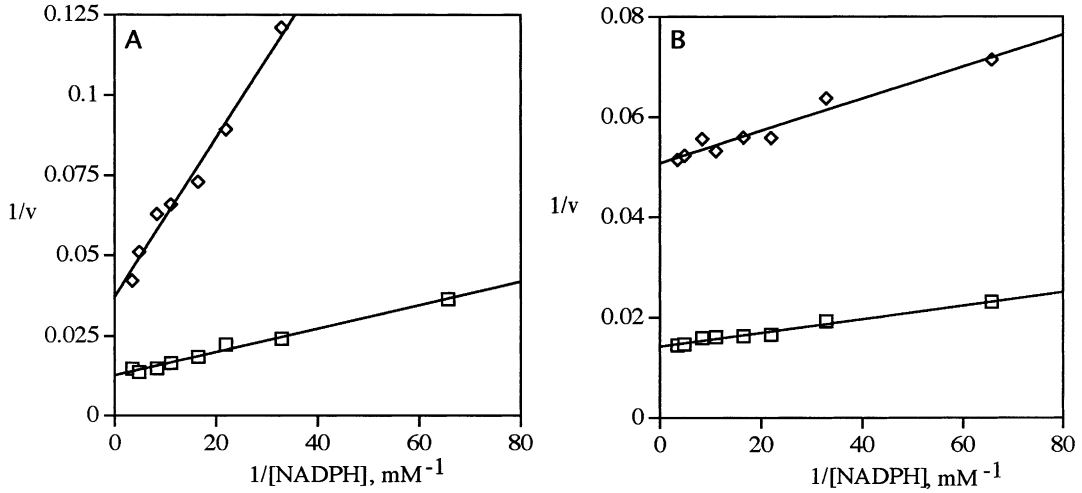

Fig. 9. Effect of the NBD-NADPH adduct on reverse transhydrogenase activities of purified enzymes from pSA2 (7.4 μg protein) (A) and pCH25 (46 μg protein) (B). □, no adduct; ◇, preincubated in dark for 30 min with 10.9 μM NBD-NADPH adduct before assay. The reverse transhydrogenase activities were assayed at different concentrations of NADPH. The concentration of AcPyAD$^+$ was 0.5 mM. *v* is expressed in nmol/min.

presence of NADPH affected the ease of binding of NADPH to the enzyme.

3.7. Formation of a transhydrogenase inhibitor by reaction of NBD chloride with NADPH

The most effective inhibition of transhydrogenase activity occurred when the enzyme was modified with NBD chloride in the presence of NADPH. This was unexpected since, as shown above, NADPH protected against modification of the β subunit by the reagent. We considered the possibility that NBD chloride reacted with the amino group of NADPH to generate an inhibitor. Fig. 7 shows the changes in the absorption spectrum with time of a mixture of NADPH and NBD chloride. NADPH and NBD chloride together showed an absorption maximum at 340–345 nm. With time this shifted towards lower wavelengths. A new absorption band developed at about 470 nm. The reaction of amino groups with NBD chloride produces an absorption band at 475 nm [13]. Thus, it is likely that the 470 nm absorption band was due to reaction of the amino group of the adenine moiety of NADPH with NBD chloride.

The ability of the compound (NBD-NADPH) formed by the reaction of NBD chloride with NADPH to act as an inhibitor of the transhydrogenase was examined. NBD-NADPH was effective in inhibiting the reverse transhydrogenase activities of the purified enzymes from pSA2 and pCH25 (Fig. 8A). NBD-NAD(H) adducts were not inhibitory. Half-maximal inhibition of reverse transhydrogenation with pSA2 and pCH25 was given by 7 and 4 μM NBD-NADPH, respectively (Fig. 8B). Cyclic transhydrogenation of AcPyAD by NADH was also effectively inhibited (Fig. 8B). NBD-NADPH was not competitive with NADPH for a binding site on the transhydrogenases from pSA2 and pCH25 (Fig. 9).

4. Discussion

The only previous study of the reaction of NBD chloride with a pyridine nucleotide transhydrogenase is that of Persson et al. [20] with the enzyme from beef-heart mitochondria. They found that the transhydrogenase was labeled by NBD chloride in a biphasic reaction with 1 or 2 cysteine residues being labeled rapidly and 3 or 4 residues more slowly. Labeling was accompanied by inhibition of transhydrogenation. All substrates of the enzyme enhanced the rate of inhibition. On irradiation with ultraviolet light at 366 nm transfer of NBD from sulfhydryl to

amino groups was observed. These workers proposed that NBD chloride reacted with sulfhydryl groups which were not at the active sites of the enzyme but which were conformationally linked to them. The specific cysteine residues labeled by the inhibitor were not determined.

The present study has extended this work and indicated that NBD chloride has several targets for interaction with the pyridine nucleotide transhydrogenase of *E. coli*. Cysteine residues were not the only amino acid residues modified by NBD chloride. The absorption spectrum of the modified enzyme showed the absorption band at about 480 nm which is characteristic of the reaction of amino groups with NBD chloride [13]. Moreover, the cysteine-free enzyme from pCH25 was inhibited by NBD chloride. Both α and β subunits reacted with NBD chloride. The α subunit was less modified than the β subunit. The tryptic cleavage pattern of the enzyme indicated that domain 1 in the α subunit was labeled. Labeling occurred in at least two regions of domain 1 since both tryptic cleavage fragments (29 and 16 kDa), resulting from scission of domain 1 at αLys-228, were labeled to some degree by the reagent. The β subunit was labeled strongly by NBD chloride in both the amino-terminal domain 2 (25 kDa tryptic fragment) and carboxyl-terminal domain 3 (30 kDa tryptic fragment) regions.

The transhydrogenase activities of the enzyme from non-mutant pSA2 were more sensitive to inhibition by NBD chloride than those of the cysteine-free enzyme from pCH25. The enzyme from pSA2 reacted with NBD chloride with modification of only two of the seven cysteine residues present in the transhydrogenase. Therefore, it is likely that modification of these two cysteine residues is associated with the increased sensitivity of the enzyme from pSA2 to the inhibition by NBD chloride. Experiments with mutant enzymes from pCH36 and pCH37 indicated that the two labeled cysteines in the transhydrogenase of pSA2 were probably βCys-147 and βCys-260. βCys-147 is located in a cytosolic loop between two transmembrane α-helices. βCys-260 is at the cytosolic end of a transmembrane α-helix [33]. βCys-147 is in a more exposed position than βCys-260. It reacts with the polar sulfhydryl-reacting biotin maleimide whereas βCys-260 does not react [34]. However, βCys-260 reacts with the non-polar *N*-(1-pyrenyl) maleimide with inhibition of transhydrogenase activity [23]. Thus, the inhibition produced by the reaction of βCys-260 with NBD chloride is analogous to that produced by *N*-(1-pyrenyl) maleimide. This cysteine residue is conserved in all known transhydrogenase sequences. It is equivalent to Cys-834 of the beef-heart mitochondrial enzyme [35]. Given the structural similarity of the transhydrogenases, it is likely that this residue was one of the 1 or 2 fast-reacting cysteine residues observed by Persson et al. [20].

A second region of modification by NBD chloride was domain 3 of the β subunit (30 kDa tryptic fragment). Modification here was prevented by NADPH. Since the presence of NADPH accelerates the inhibitory effect of NBD chloride, it is unlikely that modification of domain 3 is associated with inhibition of transhydrogenase activity. The protective effect of NADPH against modification of domain 3 by NBD chloride does not necessarily indicate that NBD chloride reacts at the NADPH-binding site. Binding of NADPH induces a conformational change in the β subunit as reflected in an increased susceptibility to proteolysis [30]. Therefore, the protective effects of NADPH may be indirect. However, reaction with NBD chloride in the presence of NADPH did affect the binding of this substrate to the modified enzyme. This was indicated during the assay of transhydrogenase activity by the induction of a lag period before maximum rates were attained. The lag was overcome by preincubation of the modified enzyme with NADPH. Since the enzyme had reacted with NBD chloride in the presence of NADPH, it is likely that the effect of modification on the binding of NADPH to its binding site in domain 3 was associated with the modification of residues in the amino-terminal domain 2 region (25 kDa tryptic fragment) of the β subunit. An analogous effect has been observed previously in mutants of the proton translocation pathway of the transhydrogenase. Residues βHis-91, βAsp-213 and βAsn-222 are located in the 25 kDa region. Mutation of these residues affected binding of NADP(H) [26,32,33] but did not provide resistance to inhibition by NBD chloride. The site(s) of interaction of NBD chloride in the 25 kDa region has not been identified. The spectrophotometric data suggest that it is likely to be an amino group(s). Mutation of the only con-

served lysine residue in this region, βLys-145, did not provide resistance to inhibition by NBD chloride (data not shown).

A third inhibitory effect of NBD chloride occurred when the enzyme was incubated with the inhibitor in the presence of NADPH. NBD chloride formed an adduct with the pyridine nucleotide. The NBD-NADPH adduct inhibited both reverse and cyclic transhydrogenation activities. Half-maximal inhibition was obtained with concentrations of the NBD-NADPH adduct less than 10μM. The NBD-NADPH adduct was not competitive with NADPH suggesting that the adduct was not reacting directly with the NADP(H)-binding site on domain 3. On the other hand, the lack of inhibition by NBD-NAD(H) adducts favors specific interaction of the NBD-NADPH adduct at the NADP(H)-binding site. It is difficult to explain these conflicting data. One possible explanation is that the NBD-NADPH adduct binds to the NADP(H)-binding site through its NADPH moiety but it also interacts with an adjacent non-polar site through its NBD moiety. If the latter interaction is sufficient to yield an inhibited enzyme, the displacement of the NADPH moiety by competing NADPH would not yield a fully reactivated enzyme. As might be expected from the structure of the adduct, there was no indication that the enzyme was covalently labelled by the inhibitor. The α and β subunits were not fluorescent following SDS-polyacrylamide gel electrophoresis of transhydrogenase which had been incubated for 2 h with the adduct.

In summary, the effect of NBD chloride on the pyridine nucleotide transhydrogenase of *E. coli* was complex. Reaction of this compound with βCys-260 inhibited the enzyme. In addition non-cysteine residues, probably lysine residues, in α and β subunits reacted with the inhibitor. Modification of domain 3 did not result in inhibition and was prevented by NADPH. Inhibition was produced by modification of the amino-terminal domain 2 region of the β subunit. This affected binding of NADPH to its binding site on domain 3. In addition, the NBD-NADPH adduct was a potent inhibitor of transhydrogenation. Thus, in experiments with NBD chloride in the presence of NADPH all of these sites of reaction will contribute to the inhibition observed. It is likely that the NBD-NADPH adduct will be more useful than NBD chloride in investigations of the pyridine nucleotide transhydrogenase.

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