J Biochem 130, 227–233 (2001)

New Inhibitors of Iron-Containing Nitrile Hydratases[1]

Didier Bonnet, Julie M. Stevens, Rodolphe Alves de Sousa, Marie-Agnès Sari, Daniel Mansuy, and Isabelle Artaud[2]

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques (UMR 8601 CNRS), Université Paris V, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France

Received February 5, 2001; accepted May 14, 2001

There is growing evidence in the literature emphasizing the significance of the post-translational modification of cysteine thiols to sulfenic acids (SOH), which have been found in a number of proteins. Crystallographic and mass spectrometric evidence has shown the presence of this group in an inactive form of the industrially important enzyme nitrile hydratase (NHase). This oxidized cysteine is unique in that it forms part of the coordination sphere of the low-spin iron III at the active site of the enzyme. The presence of this unstable sulfenic group in the active form of NHase is the subject of some controversy. To try to detect this function in NHase, we have studied the inhibitory effect on nitrile hydration of reagents known to react with sulfenic acids. Two NHases were studied, namely, *Rhodococcus rhodochrous* R312 NHase and *Comamonas testosteroni* NI1 NHase, and the reagents used were *meta*-chlorocarbonyldicyano-phenylhydrazone (m-ClCP), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), and 2-nitro-5-thiocyanato-benzoic acid (NTBA). Following this approach we report three novel inhibitors of NHases. In addition, we report thiocyanate reagents that can be used to monitor NHase activity spectroscopically.

Key words: inhibitor, NBD-Cl, nitrile hydratase, sulfenic acid, thiocyanate.

The characterization of free cysteine sulfenic acid residues (Cys-SOH) in several proteins (*1–3*), including glyceraldehyde-3-phosphate dehydrogenase (*4, 5*), AhpC phosphatase (*6*), tyrosine phosphatase (*7*), and peroxiredoxin (*8*), has revealed the potential significance of sulfenic acid chemistry in biology. Only one example of a sulfenic acid coordinated to a metal ion has been reported, namely, in the active center of the nitrosylated form of nitrile hydratase (*9*). Nitrile hydratases (NHases) are bacterial non-heme Fe^{III} or non-corrinoid Co^{III} enzymes that consist of an $\alpha_2\beta_2$ tetramer and catalyze the specific hydration of nitriles into the corresponding amides. Fe-type enzymes can exist in an active non-nitrosylated form, or an inactive nitrosylated form that can be reactivated by light irradiation (*9–11*). The X-ray structure of the inactive nitrosylated form of the iron-containing NHase from *Rhodococcus* species N771 (*9*) shows that all of the residues involved in binding the metal are found in a small consensus sequence of six amino acids, Cys_{109}-Ser-Leu-Cys-Ser-Cys_{114}. This sequence is located in the α subunit of the protein and is highly conserved among all iron-containing NHases. In this structure, the iron is coordinated to two amide nitrogens (from the peptide backbone of Cys_{114} and Ser_{113}), a nitrosyl ligand and three sulfurs from cysteines 114, 112, and 109. Cys_{114} and Cys_{112} are post-translationally oxidized to sulfenic and sulfinic acids respectively. The structure of the coordination sphere of the inactive protein is shown in Fig. 1. These oxidation states were not observed in the earlier X-ray structure of the R312 iron NHase (which has an identical amino acid sequence to N771) due to the low resolution (*12*). Mass spectrometry experiments have clearly established the presence of the S-coordinated sulfinic (SO_2H) group in both the active and inactive forms (*13–15*), which is in agreement with the differential FTIR vibrations observed at 1020 and 1150 cm-1 during photoactivation of N771 NHase (*16*). The presence of the S-coordinated sulfenic acid residue (SOH), however, in the active form of iron NHase remains controversial. This oxidation state has been observed by mass spectrometry and X-ray crystallography in the inactive form (*9*). However, no SO vibration expected around 900 cm^{-1} is present in the differential FTIR spectrum of the protein (*16, 17*).

In the case of peroxiredoxin and hydroperoxide reductase, the free sulfenic acid has been proposed to play a redox role in catalysis (*3*). For the oxidized form of glyceraldehyde-3-phosphate dehydrogenase, however, nucleophilic attack by the sulfenic acid has been suggested to account for the observed acyl phosphatase activity (*1, 4*). If a sulfenic group is present in the NHase active site, such a reactivity of the coordinated sulfenate residue might also be proposed for NHase catalysis. In order to obtain indirect evidence of the presence of a sulfenic group in the active site of NHase and to test its potential involvement in the

[1] This work was funded by the European Commission TMR-NOHE-MIP research network N° ERBFMRXCT98-0174

[2] To whom correspondence should be addressed Tel: +33-1-42 86 21 89, Fax. +33-1-42 86 83 87, E-mail artaud@biomedicale.univ-paris5.fr

Abbreviations NHase, nitrile hydratase; m-ClCP, carbonyl cyanide 3-chlorophenylhydrazone; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NTBA, 5-nitro-3-thiocyanato-benzoic acid, DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid), R312, NHase from *Rhodococcus rhodochrous* R312, NI1, NHase from *Comamonas testosteroni* NI1; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.
Proteins and enzymes nitrile hydratase [EC 4 2.1.84].

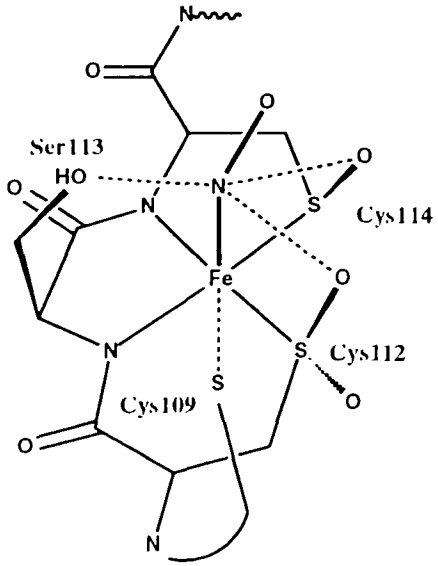


Fig 1. **Representation of the structure of the Fe coordination sphere of N771 nitrile hydratase.** The NO coordinated to the Fe is shown as well as the post-translationally modified cysteines 112 and 114

catalytic mechanism of nitrile hydration, we examined the effect of several compounds reported in the literature to react with protein or non-protein free sulfenic acids. Usually, inhibitors are a good tool for the understanding of a reaction mechanism. Two NHases were studied, namely, *Rhodococcus rhodochrous* R312 NHase, for which the structure is known (*9*), and *Comamonas testosteroni* NI1 NHase (*18*), for which the structure is unknown (although the protein has a 50% sequence identity with R312 NHase). Based on this approach we have found three new, potent inhibitors of NHases.

MATERIALS AND METHODS

Unless otherwise specified, all experiments were performed in 100 mM HEPES-KOH buffer, pH 7.2 (HEPES buffer). UV-visible spectra were recorded on a Uvikon 820 double-beam spectrophotometer, with a 1-cm path length and at a temperature of 6–10°C. All reagents were purchased from Aldrich and were of the highest analytical grade. Cyanovaleric acid and adipamic acid were provided by Rhône-Poulenc.

Purification of Nitrile Hydratase—R312 and NI1 NHases were purified from the original bacterial strains and stored in the presence of 40 mM butyric acid in HEPES buffer as previously described for R312 (*19*) and according to the procedure described in an industrial patent for NI1 (*18*).

Enzyme Assays—NHase activity was assayed by following the hydration of acrylonitrile for the R312 enzyme, and 5-cyanovaleric acid for the NI1 enzyme. Solutions of 1 ml of substrate (17 mM acrylonitrile for R312, and 2.5 mM 5-cyanovaleric acid for NI1) were incubated with 1.30 nM enzyme at 28°C for 10 min. Amide production versus time was linear for 10 min. The reaction was stopped by the addition of 1 ml of 0.1 N HCl. The amount of acrylamide or adipamic acid formed in the reaction mixture was monitored by analytical HPLC. HPLC was performed on a Gilson system equipped with a Nucleosil 5 mm C18 column (reverse-phase column, 4.6 × 250 mm) at a flow rate of 1 ml/min using a mixture of acetonitrile and 5 mM H_3PO_4 (pH 2.4) 5/95 (v/v) as the solvent, and with detection at a wavelength of 208 nm.

Determination of Inhibition Constants—IC_{50} values were determined by performing enzyme assays as described above and in the presence of different concentrations of the inhibitors. The IC_{50} is the inhibitor concentration at which half of the enzyme activity remains. The K_i values were determined by measuring the reaction velocity at different concentrations of substrate and different concentrations of the reagents The results were analyzed using the standard double-reciprocal plot analysis as well as secondary plots of the slopes versus the inhibitor concentrations. Acetone and dimethyl sulfoxide (when NI1 and R312 were used, respectively) were used as solvents for NBD-Cl, m-ClCP, and dimedone, and the final volume of solvent did not exceed 2% in the final assay mixture. The solvents were shown to have no effect on the enzyme activity at this concentration. Solutions of the other reagents were prepared in HEPES buffer.

Conversion of m-ClCP by NI1 NHase—One milliliter of 0.5 mM m-ClCPcontaining different concentrations of NI1 NHase (up to 40 nM) was incubated for different times (up to 60 min) at 28°C. The reaction was stopped by the addition of 1 ml of acetonitrile The conversion of m-ClCP to 2-[(3-chlorophenyl)-hydrazono]-2-cyano-acetamide was monitored by analytical HPLC on a Nucleosil 5 mm C18 column (4.6 × 250 mm) at a flow rate of 1 ml/min A 20-min gradient of 30–80% acetonitrile/5 mM H_3PO_4, pH 2.4 was used for elution. The retention times were 14.5 min for m-ClCP and 8 3 min for 2-[(3-chlorophenyl)-hydrazono]-2-cyano-acetamide. The retention time for the product was verified by comparison with the retention time of the authentic product, synthesized as described below.

Synthesis of 2-[(3-Chlorophenyl)-Hydrazono]-2-Cyano-Acetamide—2-[(3-Chlorophenyl)-hydrazono]-2-cyano-acetamide was synthesized as previously described (*20*). $C_9H_7ClN_4O$ (222.6 g/mol). NMR ^1H [250 MHz; DMSO d^6; δ(ppm)]: 11.73 (NH, s, 1H); 7.95 (NH_2, s, 1H); 7.81 (H_{ar}, s, 1H); 7.55 (H_{ar}, m, 1H); 7.48 (NH_2, s, 1H); 7 35 (H_{ar}, t, 1H); 7 11 (H_{ar}, m, 1H). IR (KBr pellets) ν (CN) 2220 cm^{-1}. Elemental analysis (Exp) C 47.23; H 3.33; N 24.30 (%); (Calc $C_9H_7ClN_4O$, 0.35 H_2O) C 47.22; H 3.39; N 24.47 (%).

NBD-Cl Titration of NI1 NHase—NI1 NHase was diluted to a final concentration of 35 μM in 100 μl of 50 mM phosphate buffer pH 7.2 containing 2 M guanidine hydrochloride to denature the protein. Ten equivalents of NBD-Cl were added and the solution was then incubated for 15 min at 4°C. After the reaction, the excess NBD-Cl and guanidinium chloride were removed from the protein by five successive centrifugations at 4°C in an Amicon Centricon 10,000 (diluting the protein after each concentration step in 50 mM phosphate buffer pH 7.2). The modified protein was analyzed by UV-vis spectroscopy.

Preincubation of NI1 NHase with NBD-Cl—NI1 was preincubated at 260 nM with NBD-Cl at 50 and 100 μM for 0, 2, 4, 6, 8, or 10 min. A 5-μl aliquot was then diluted into 1 ml of cyanovaleric acid (2.5 mM) for enzyme assay, as previously described. A blank experiment was performed under the same conditions, in the absence of NBD-Cl.

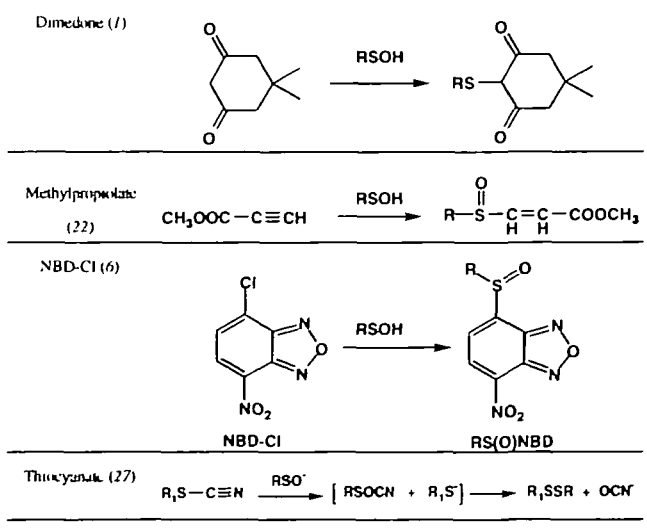
Synthesis of 2-Acetylamino-3-(7-Nitro-Benzo[1,2,5]Oxadi-

azole-4-Sulfanyl)-Propanoic Acid Methyl Ester—Powdered NaH (40.5 mg, 1.69 mmol) was added to a tetrahydrofuran solution of *N*-acetyl cysteine methyl ester (300 mg, 1.69 mmol in 20 ml). The solution darkened after addition of powdered NBD-Cl (337 mg, 1.69 mmol) and was then immediately evaporated to dryness. The residue was dissolved in CH_2Cl_2, and after eliminating the NaCl by filtration, the thiol adduct crystallized upon evaporation of the solvent (150 mg, 26% yield). $C_{12}H_{12}N_4O_6S$ (340 g/mol). NMR ^1H [250 MHz; $CDCl_3$; δ(ppm)]. 8.41 ($1H_{ar}$, d, J = 7.5 Hz); 7.45 ($1H_{ar}$, d, J = 7.5 Hz); 6.34 (NH, d); 4.96 (1H, CH, m); 3.78 (5H, OMe, CH_2, m), 2.02 (3H, s). UV-vis in EtOH (λ nm, ε M^{-1} cm^{-1}) 415 (12,000). Mass (CI): m/z 241, 75%, $[M + H]^+$; 321, 100%, $[M–OMe+ 2H]^+$.

Synthesis of 2-Acetylamino-3-(7-Nitro-Benzo[1,2,5]Oxadiazole-4-Sulfinyl)-Propanoic Acid Methyl Ester—A stock solution of dimethyl dioxirane in acetone (0.1 N, 100 ml) was prepared and titrated by oxidation of methylphenylsulfide to the corresponding sulfoxide as previously described (*21*) Dimethyl dioxirane (0.32 mmol) was added to an acetone solution of the thiol adduct (100 mg, 0.29 mmol in 25 ml) and cooled to –45°C. After stirring for 1 h at this temperature, the solution was slowly heated to room temperature. The acetone was then evaporated under vacuum at 0°C and the yellow powder was purified by column chromatography (SiO_2, CH_2Cl_2/Acetone, 80:20). Two fractions were collected: the first containing the sulfone and the second the sulfoxide (25 mg, 25% yield). $C_{12}H_{12}N_4O_7S$ (356 g/mol) NMR ^1H (250 MHz; $CDCl_3$; δ(ppm)]: 8.61 ($1H_{ar}$, d, J = 7.5 Hz); 8.14 ($1H_{ar}$, d, J = 7.5 Hz); 7.45 (NH, m); 5.05 (1H, CH, m); 3.6–4 1 (2H, CH_2, m); 3.81 (3H, OMe, s); 2.01 (3H, s). UV-vis in EtOH (λ nm, ε M^{-1} cm^{-1}): 350 (6,100) Mass (CI): m/z 357, 100%, $[M + H]^+$.

Thiocyanate Transformation by NHases—NTBA (1 mM) in HEPES buffer was incubated with different amounts of NI1 enzyme (up to 80 nM) at 4°C for 25 min The appearance of the thiolate product was monitored by the absorption at 412 nm. This wavelength is routinely used for thiolate detection (absorption coefficient 13,600 M^{-1} cm^{-1}) in titrations of free cysteine residues with Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid), DTNB]. The transformation of ethyl thiocyanate by R312 NHase was monitored by the addition of a twofold excess of DTNB after 30 min of incubation of the enzyme with the thiocyanate (final concentrations of 8.3 nM and 200 μM respectively) at 4°C. The absorption change at 412 nm after a further 40 min was used to monitor the amount of thiolate produced by the enzyme from the thiocyanate.

TABLE I **Some known reactions of sulfenic acid reagents.**



RESULTS AND DISCUSSION

Effect of Dimedone and Methyl Propiolate on NHase Activity—Dimedone (*1*) and methyl propiolate (*22*) are reagents that are known to react with free sulfenic acid groups (Table I) For example, dimedone has been used to show the presence of a sulfenic group in the protein cathepsin K (*23*). Inhibition studies of these products with NHases revealed no inhibitory effect in either case (as shown in Table II). A possible explanation for why dimedone and methyl propiolate did not inhibit the enzyme is that the coordination of the sulfenate to the iron in the NHase active site prevents any reaction with these reagents.

Influence of m-ClCP on NHase Activity—m-ClCP [$mClC_6H_4NHN{=}C(CN)_2$] has been described as an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (*4*), which has a sulfenic group at its active site (*1, 4*). As m-ClCP contains two cyano groups, we considered that it might be recognized by NHases and so tested it as an inhibitor of R312 and NI1 NHases. Figure 2 shows the double-reciprocal plot of the reaction velocity of NI1 NHases *versus* substrate concentration at different concentrations of m-ClCP. This result shows that the inhibition is competitive and indicates

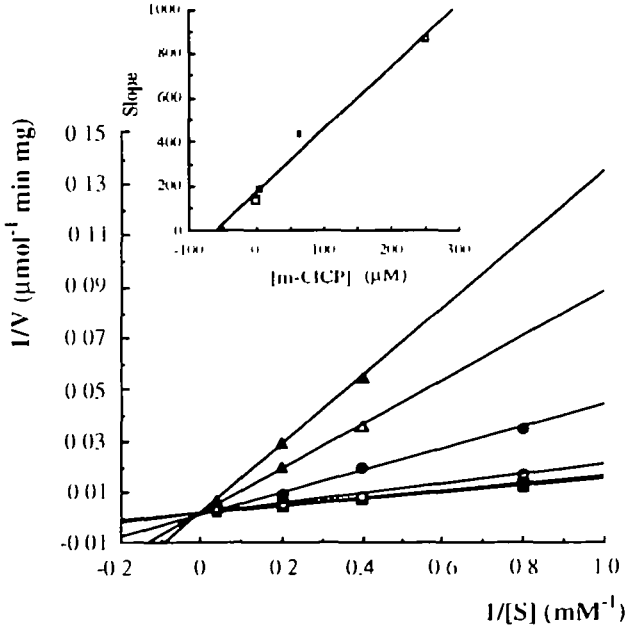


Fig 2 **Competitive inhibition of NI1 nitrile hydratase by m-ClCP.** Double-reciprocal plots of the rate of adipamic acid formation *versus* cyanovaleric acid concentration at different m-ClCP concentrations (■) 0 24 μM, (○) 3 9 μM, (●) 62 5 μM, (△) 250 μM, (▲) 1,000 μM. Experiments were performed with 1.3 nM NHase in 100 mM HEPES pH 7.2 containing the appropriate amount of substrate at 28°C for 10 min The inset shows the secondary plot of the line slopes *versus* m-ClCP concentration

that m-ClCP interacts with the enzyme at the active site. The secondary plot of the slopes versus the inhibitor concentrations shown in the inset gives an intercept on the *x*-axis yielding an inhibition constant of 50 μM, as shown in Table II. In order to establish whether the enzyme recognized m-ClCP as a substrate, a sample of enzyme was incubated with an excess of m-ClCP and HPLC analysis was performed on the reaction mixture to detect the amide derivative. The hydrated product 2-[(3-chlorophenyl)-hydrazono]-2-cyano-acetamide was synthesised to provide an authentic sample for HPLC. It was found that m-ClCP was hydrated by the enzyme and the maximum conversion of the nitrile to the amide form was approximately 4%, using 0.5 mM m-ClCP and 16 nM NI1 NHase over 60 min at 28°C (data not shown). In addition, m-ClCP was found to be a competitive inhibitor of R312 NHase with a K_i value of 65 μM, as shown in Table II. This compound, therefore, has a good affinity for both enzymes and competes with the substrate for the active site. We have also shown that it is recognized as a substrate, although the level of conversion to the amide form is low, explaining its reasonably good inhibitory effect. It behaves in the same manner as isobutyronitrile, a well-known competitive inhibitor of R312 NHase (K_i = 5.4 μM) (*19*), that is also recognized as a substrate.

TABLE II **Inhibition constants of the sulfenic acid reagents for NHases.** K_i and IC_{50} values were determined as described in "MATERIALS AND METHODS," and are mean values ± SD of at least three experiments. (nd = not determined)

Inhibitor	NHase R312 K_i (μM)	NHase NI1 K_i (μM)
m-ClCP	65 (± 10)	50 (± 5)
NBD-Cl	1 (± 0 5)	3 (± 0 5)
NTBA	IC_{50} > 4 mM	IC_{50} = 20 (± 2)
EtSCN	IC_{50} = 45 μM (± 5)	nd
Dimedone	nd	IC_{50} > 1 mM
Methyl propiolate	nd	IC_{50} > 10 mM

Inhibition of NHases by NBD-Cl—As NBD-Cl is the reagent of choice to detect sulfenic groups, we tested it as an inhibitor of nitrile hydration catalyzed by R312 and NI1 NHases. It was found to be the best inhibitor reported so far for NHases, with inhibition constants of 1 μM for R312 and 3 μM for NI1 (Table II). An inhibitor with a K_i in this range is a good inhibitor of NHases, because these enzymes have K_m values for their substrates in the mM range. The double-reciprocal plots for both NHases, and the secondary plots in the inset, are shown in Fig. 3. According to these plots, the inhibition appears to be non-competitive, suggesting that NBD-Cl interacts with the protein at a site other than that of the substrate. Whether it interacts directly with any part of the active site or not is difficult to conclude on the basis of these results.

NBD-Cl is known to form an adduct with sulfenic acids, exhibiting a specific absorption in the visible spectrum at 347 nm (*6*). Given its electrophilic nature, NBD-Cl also reacts with other nucleophiles in proteins such as cysteine, tyrosine or lysine residues, producing the conjugates CysS-NBD, TyrONBD, and LysNHNBD, which have characteristic absorption maxima at 420, 480, and 382 nm respectively (*6*). The structure of the sulfenic adduct has never been fully characterized and has been suggested to be a sulfoxide or a sulfenic ester depending on whether the sulfur or the oxygen of the sulfenate acts as the nucleophilic center. To further characterize this adduct, we prepared the sulfoxide RS(O)NBD derived from *N*-acetyl-cysteine methyl ester (see "MATERIALS AND METHODS") The RSONBD adduct exhibited an absorption maximum at 350 nm as the sulfenic adduct in a protein (*6*), supporting the hypothesis that the adduct is a sulfoxide rather than a sulfenic ester. However, we cannot exclude the possibility that the sulfoxide results from the spontaneous rearrangement of a transient sulfenic ester (*24, 25*)

NBD-Cl was found to be a reversible inhibitor of NI1

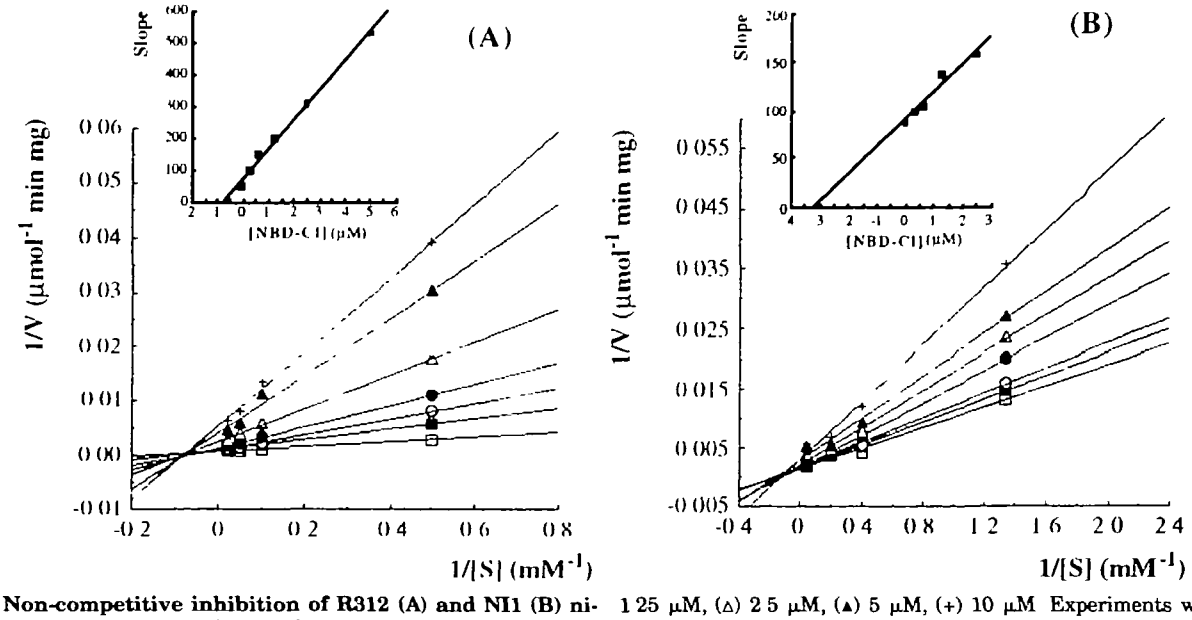


Fig. 3. **Non-competitive inhibition of R312 (A) and NI1 (B) nitrile hydratases by NBD-Cl.** Double-reciprocal plots of the rate of (A) acrylamide formation *versus* acrylonitrile concentration or (B) adipamic acid formation *versus* cyanovaleric acid concentration at different NBD-Cl concentrations: (□) 0 μM, (■) 0.31 μM, (○) 0 62 μM, (●) 1 25 μM, (△) 2 5 μM, (▲) 5 μM, (+) 10 μM Experiments were performed with 1 3 nM NHase in 100 mM HEPES pH 7 2 containing different amounts of substrate at 28°C for 10 min. The inset shows the secondary plot of the line slopes versus NBD-Cl concentration.

NHase, since preincubation of the enzyme with this inhibitor at 50 or 100 μM for up to 10 min did not lead to a significant loss of activity in comparison with a blank experiment performed with the enzyme alone However, preincubation experiments were performed by incubating a concentrated enzyme solution (260 nM) with NBD-Cl, followed by dilution into assay buffer. The experiment was performed in this way because, under dilute conditions and in the absence of substrate, the enzyme loses its activity very rapidly.

Finally, an UV-vis spectroscopic study of the reaction of native NI1 with NBD-Cl failed to detect the formation of any adduct. It is possible, though, that an Fe-SONBD adduct would be unstable and would be cleaved upon reaction with water. For example, a Co^{III} *S*-coordinated methyl sulfenic ester, prepared by *O*-alkylation of the *S*-coordinated sulfenate with methyl iodide, yields an unstable coordinated sulfoxide (*26*).

However, reaction of the protein with NBD-Cl in the presence of 2 M guanidine hydrochloride (to denature the protein), followed by removal of the excess NBD-Cl, resulted in a maximum absorption at 420 nm as shown in Fig. 4. An absorption at this wavelength suggests that a CysS-NBD adduct has been formed. On the basis of the reported molar absorption of 13,400 M^{-1} cm^{-1} (*6*), it was calculated that 1.5 ± 0.2 cysteines were titrated. Therefore, of the 8 cysteines present in the NI1 protein (5 in the α subunit and 3 in the β subunit) (*18*) only 1 or 2 seem to be accessible to this reagent.

In conclusion, no sulfenic adduct was detected. Either no sulfenic group is present in the active site, or the sulfenic group is present and, under denaturing conditions, the iron would be released into solution and the resulting free sulfenic moiety would react rapidly with the non-oxidized cysteine of the iron coordination sphere, producing a disulfide, before reaction with NBD-Cl could occur.

Influence of Thiocyanates on NHase activity—Inhibition of NHases by thiocyanates: Thiocyanates are proposed to react with sulfenates to produce a disulfide and an isocyanide, according to the mechanism shown in Table I (*27*). As NI1 NHase is known to recognize compounds containing carboxylic acids (*18*), the reagent 2-nitro-5-thiocyanatobenzoic acid (NTBA) was selected as a potential inhibitor of this enzyme. As shown in Table II, NTBA is a good inhibitor of NI1 NHase, with an IC_{50} of 20 μM. The double-reciprocal plot suggests that the inhibition is non-competitive, but the secondary plot shows a non-linear relationship between the slope and the inhibitor concentration (data not shown). This suggests that the inhibition is more complex, and could be of a mixed hyperbolic type (*28*).

The same thiocyanate is a very weak inhibitor of R312 NHase, with an IC_{50} greater than 4 mM. The difference in the specificity of this reagent for the two enzymes emphasizes their different affinities for molecules containing a COOH group. The aliphatic thiocyanate ethyl thiocyanate, which resembles the substrate acrylonitrile more closely, is however a good inhibitor of R312 NHase with an IC_{50} of 45 μM.

According to these results thiocyanates might be substrates of NHase.

Use of thiocyanates as a spectroscopic probe to monitor NHase activity: There are two pathways by which the enzyme could react with thiocyanates that are recognized as substrates. One possible pathway is the formation of the hydrated product $RSCONH_2$, and the other is the release of the thiolate following a mechanism that could be similar to that described in Table I, assuming the existence of a sulfenic group in the active site. The thiolate product would be 2-nitro-5-mercaptobenzoic acid, whose formation could be monitored by the absorption change at 412 nm. In fact, this thiolate corresponds to the product of the reaction between DTNB and sulfydryl groups, a reaction that is routinely used to estimate the number of cysteines in a protein spectroscopically An increase in the absorption at 412 nm was observed during incubation of NI1 NHase with NTBA, as indicated by the enzyme dependence of this absorption change, shown in Fig 5. The inset in Fig. 5 shows the absorption spectrum of the product, with the maximum absorption at a wavelength of 412 nm. A slight production of the thiolate was observed in the HEPES buffer in the absence of enzyme, and the results shown were corrected for this. These results demonstrate that the conversion of

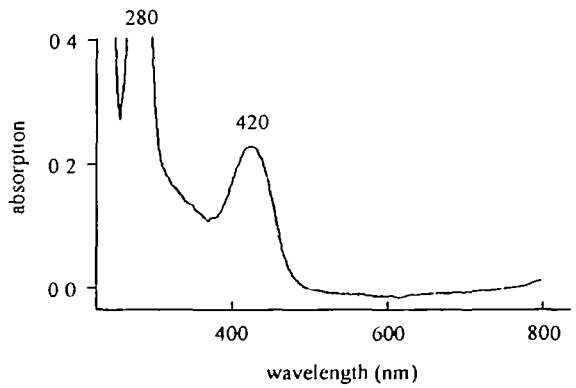


Fig. 4. **UV-visible spectrum of NI1 nitrile hydratase incubated with NBD-Cl.** The enzyme (35 μM) was treated with 10 molar equivalents of NBD-Cl in the presence of 2 M guanidinium chloride in 50 mM phosphate buffer pH 7 2, followed by removal of excess NBD-Cl

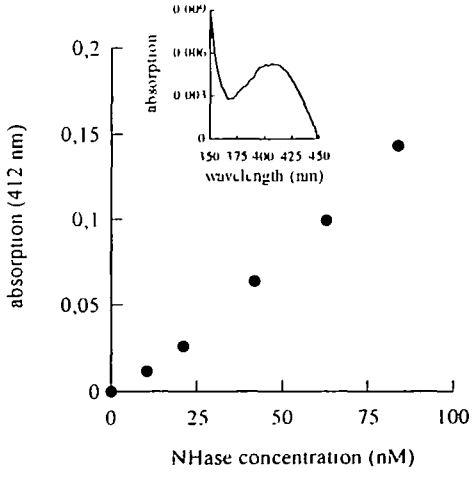


Fig. 5. **Enzyme-dependent conversion of NTBA by NI1 NHase.** The conversion was monitored by the absorption at 412 nm after a 25-min incubation of NI1 NHase with 1 mM NTBA in 100 mM HEPES pH 7 2. Blank values have been subtracted. The inset shows the absorption spectrum of the product, with a maximum absorption at 412 nm

the thiocyanate can be related directly to the amount of enzyme, and that this process can be monitored spectroscopically. We have also shown that it is possible to use any thiocyanate that is recognized by the enzyme as a spectrophotometric probe. By incubating R312 NHase with ethyl thiocyanate (final concentrations of 8.3 nM and 200 μM respectively) and then adding a two-fold excess of DTNB, it was possible to monitor the appearance of the thiolate by the absorption at 412 nm, which corresponds to the titration of EtS^- with DTNB (results not shown) The percentage conversion of ethyl thiocyanate was 2.7% under these conditions. The percentage conversion for NTBA by NI1 NHase was 1.1% for the results shown in Fig. 5. In both cases the level of conversion is low, but the change can be easily monitored because of the strong absorption properties of the thiolate product It was not possible to determine the kinetic constants for the thiocyanates due to high rates of spontaneous conversion at high thiocyanate concentrations. Thiocyanates could provide, as methacrylonitrile (*14, 15*); a more rapid and convenient method for assaying the enzyme, particularly during enzyme purification. The hydration of nitriles followed by HPLC, however, gives a more accurate determination of enzyme activity. In addition, the specificity of the thiocyanate reagents (RSCN) can be controlled by changing the R group to suit the properties of the active site of the enzyme.

Conclusion—In this study, we have described the effect on the catalytic activity of nitrile hydratases of reagents known to irreversibly inhibit sulfenic-containing proteins. However, the specificity of these compounds toward sulfenic groups has not been definitively established. NBD-Cl, for example, gives rise to the formation of adducts with any nucleophile in proteins. Moreover, while the reactivity of dimedone, methylpropiolate and thiocyanates is well understood with non-protein free sulfenic acids, and that of NBD-Cl with free sulfenic acids in proteins, we do not have any information concerning either the reactivity of these reagents with *S*-coordinated sulfenic moieties, or the stability of a hypothetical *S*-coordinated sulfenic oxygen adduct. Thus the fact that some of these reagents inhibit NHase does not necessarily imply that they function at a sulfenic moiety, and no definitive conclusion can be drawn on the presence of a sulfenic group at the enzyme active site. It is possible that the observed inhibition by NBD-Cl and thiocyanates could arise from a reaction of these compounds with the hydroxy coordinated to the iron active site. Nevertheless, following this strategy, we found three novel iron-NHase inhibitors of high affinity, namely, m-ClCP, NBD-Cl, and thiocyanates. NBD-Cl is the best inhibitor reported so far for NHases. m-ClCP and thiocyanates are recognized by the enzyme as substrates, and thiocyanates can be used in a spectroscopic assay of the enzyme, which could provide a more convenient method for rapid determination of activity.

The authors thank Dominique Pétré from Rhone-Poulenc for providing the NHase-producing bacterial strains. D.B gratefully acknowledges Rhone-Poulenc for a Ph D fellowship and J.S. acknowledges the European Commission for a post-doctoral fellowship.

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