Mammalian Thioredoxin Reductase Is Irreversibly Inhibited by Dinitrohalobenzenes by Alkylation of Both the Redox Active Selenocysteine and Its Neighboring Cysteine Residue*

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The immunostimulatory dinitrohalobenzenes compound 1-chloro-2,4-dinitrobenzene (DNBC) irreversibly inhibits mammalian thioredoxin reductase (TrxR) in the presence of NADPH, inducing an NADPH oxidase activity in the modified enzyme (Arner, E. S. J., Björnstedt, M., and Holmgren, A. (1995) J. Biol. Chem. 270, 3479–3482). Here we have further analyzed the reactivity with the enzyme of DNBC and analogues with varying immunomodulatory properties. We have also identified the reactive residues in bovine thioredoxin reductase, recently discovered to be a selenoprotein. We found that 4-vinylpyridine competed with DNBC for inactivation of TrxR, with DNBC being about 10 times more efficient, and only alkylation with DNBC but not with 4-vinylpyridine induced an NADPH oxidase activity. A number of nonsensitizing DNBC analogues neither inactivated the enzyme nor induced any NADPH oxidase activity. The NADPH oxidase activity of TrxR induced by dinitrohalobenzenes generated superoxide, as detected by reaction with epinephrine (the adrenochrome method). Addition of superoxide dismutase quenched this reaction and also stimulated the NADPH oxidase activity. By peptide analysis using mass spectrometry and Edman degradation, both the cysteine and the selenocysteine in the conserved carboxyl-terminal sequence Gly-Cys-Sec-Gly (where Sec indicates selenocysteine) were determined to be dinitrophenyl-alkylated upon incubation of native TrxR with NADPH and DNBC. A model for the interaction between TrxR and dinitrohalobenzenes is proposed, involving a functional FAD in the alkylated TrxR generating an anion nitroradical in a dinitrophenyl group, which in turn reacts with oxygen to generate superoxide. Production of reactive oxygen species and inhibited reduction of thioredoxin by the modified thioredoxin reductase after reaction with dinitrohalobenzenes may play a major role in the inflammatory reactions provoked by these compounds.

TrxR catalyzes the NADPH-dependent reduction of the active site disulfide in oxidized thioredoxin to a dithiol in reduced thioredoxin. Thioredoxin is an ubiquitous 12-kDa protein with a large number of biological activities (1–6). Reduced thioredoxin is a powerful protein disulfide reductase catalyzing electron transport to ribonucleotide reductase and other reductive enzymes or redox regulation of enzymes and transcription factors. Secreted thioredoxin has cytokine-like effects on certain mammalian cells (2–7).

Thioredoxin reductase from Escherichia coli has been extensively characterized (1), and a high resolution x-ray structure shows surprisingly large differences to the other members of the pyridine nucleotide-disulfide oxidoreductase family (8, 9). Thus, the subunits of about 35 kDa are smaller than the about 50-kDa subunits present in glutathione reductase from all species. Furthermore the active site cysteine residues of E. coli TrxR are located in the central NADPH domain and separated by two amino acids (Cys-Ala-Thr-Cys), in comparison with the active site in glutathione reductase which is Cys-Val-Asn-Val-Gly-Cys and located in the NH2-terminal FAD domain, suggesting convergent evolution (9). The structural features of TrxR from E. coli with a high specificity for its homologous Trx are also typical for TrxR from prokaryotes, lower eukaryotes like yeast, or plants (1, 8–10).

It has long been known that mammalian TrxR has properties strikingly different to the enzyme from E. coli and lower organisms (2–4). The enzymes from calf liver and thymus and rat liver were first purified to homogeneity and showed 58-kDa subunits (11, 12). The mammalian thioredoxin reductases, including that of human placenta (13), are thereby larger than the E. coli enzyme and in contrast have a wide substrate specificity. Thus, the mammalian enzymes will reduce thioredoxins from different species (11), several low molecular weight disulfide substrates including DTNB used in assays (11, 12) or lipidic acid (14) as well as other nondisulfide substrates including selenoglutathione (15), selenite (16), alkoxen (17), or most surprising lipid hydroperoxides (18). The wide substrate specificity indicates an unusual structure of the active site, which is also demonstrated by the inhibition of mammalian TrxR by several drugs in clinical use including antitumor quinones (19, 20), nitrosoureas (21), or 13-cis-retinoic acid (22).

Recently, two proteins from transformed human cells having thioredoxin reductase activity, a 55-kDa subunit enzyme from a Jurkat T-cell line (23) and a 57-kDa subunit protein from a lung adenocarcinoma cell line (24), were shown to contain selenocysteine. A peptide sequence from the protein purified from the human T-cell line (23) agreed with a putative human dinitrobenzene; dnp, dinitrophenyl; 2,5-DCNB, 2,5-dichloronitrobenzene; 3,4-DCNB, 3,4-dichloronitrobenzene; 4-VP, 4-vinylpyridine; IAA, iodacetatic acid; Sec or U, selenocysteine; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HPLC, high performance liquid chromatography.
Fig. 1. Domain organization of mammalian TrxR. The proposed domains of a TrxR subunit are based on the amino acid sequence of rat, bovine, and human TrxR (26). Mammalian TrxR has close to 500 amino acid residues, and the active enzyme is a homodimer. The FAD and bovine, and human TrxR (26). Mammalian TrxR has close to 500 amino acid domains as well as the central and interface domains indicated in the figure are suggested by the high homology to glutathione reductase (1, 26). The sequences in the NH₂-terminal FAD domain encompassing the active site disulfide motif identical to that of GR and the carboxyl-terminal elongation with the penultimate Sec and its neighboring Cys are shown by one-letter amino acid abbreviations. These motifs are conserved between the mammalian species (26).

The mammalian TrxR amino acid sequence is highly homologous to GR and carries the identical sequence motif within the NH₂-terminal FAD domain that contains the redox active cysteine residues of GR. The carboxyl-terminal motif with a penultimate Sec and a neighboring Cys residue is an elongation to the structure that is not present in GR, but is homologous to a Cys-Cys-containing elongation found in another related flavoprotein, mercuric reductase (26). The domain organization of TrxR is schematically depicted in Fig. 1. DNBC is an electrophilic compound used as a substrate in assays to determine glutathione S-transferase, which is involved in elimination of DNBC in vivo (27). DNBC is therefore also used in cell culture experiments as a GSH-depleting agent (28). Furthermore, DNBC has an established use as an immunomodulatory agent to provoke delayed-type hypersensitivity (29). Although proposed to function as a hapten, the mechanism of DNBC immunomodulation is not clear (30).

To summarize our earlier findings, we showed that DNBC irreversibly inhibited mammalian TrxR with second order kinetics by alkylating the enzyme, but that TrxR had to be reduced by preincubation with NADPH for the alkylation to occur (31). Upon alkylation, an NADPH oxidase activity could be detected, which was about 30-fold increased as compared with that of the native enzyme, and no consumption of NADPH was seen under anaerobic conditions (31). In the present study we demonstrate that the nitro groups of the dinitrophenyl-alkylated TrxR are necessary for the induction of the NADPH oxidase activity, and we identify the residues alkylated by DNBC.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—DNBC, DNFB, DNBC analogues, IAA, and 4-VP as well as epinephrine bitartrate salt and SOD were purchased from Sigma. Mammalian TrxR was purified from human placenta and calf thymus as described (12). Enzyme concentration was determined from the absorbance at 340 nm using a molar extinction coefficient of 12000 M⁻¹ cm⁻¹ (32).

Identification of dnp-alkylated Amino Acid Residues—TrxR (75 µg, ~1.3 nmol of subunit) was preincubated for 30 min at room temperature with NADPH (250 nmol) in 15 µl of 50 m Tris-Cl, 2 mM EDTA, pH 7.5. Then 0.5 µl of 260 mM DNBC in ethanol was added to give 130 nmol (TrxR subunit). DNBC = 1·100 nmol, 3% ethanol), and alkylation was allowed to proceed for 20 min at room temperature. Then the sample was put on ice and subsequently run on a Fast desalting PC 3.2/10 column equilibrated with 50 mM Tris-Cl, 2 mM EDTA, pH 7.5, using the SMART HPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden). To the alkylated enzyme guanidine hydrochloride (final concentration: 0.8 m) and 7.5 µg of Lys-C endopeptidase (Wako) was added and the sample was incubated for 17 h. Then a further additional 2.5 µg of Lys-C endopeptidase was added and incubation continued for 1.5 h at 37 °C. Then peptides were separated by automated fractionation using the SMART HPLC system with a Sepasil µRPC C2/C18 2.1/10 column in a gradient of 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B) using 100% buffer A for 6 min, a linear gradient to 50% buffer B in 120 min, followed by a linear gradient to 100% buffer B in 3 min and finally 100% buffer B for 15 min. Peptides were then identified by absorbance at 214 and 254 nm, while dnp-alkylated peptides were identified by absorbance at 340 nm. Fractions with absorbance at 340 nm were taken to determine the amino acid sequence determination using Edman degradation on a Procise Protein Sequencer (Applied Biosystems) or to analysis using electrospray mass spectrometry with an AutoSpec QATOF/FFD (Micromass, Manchester, UK). Yields at the individual cycles of the Edman degradation were as follows (in picomoles): peptide in fraction B, 4.7·10³-2.5·10³-7.3·10⁻¹-11·10⁻⁹, 9·10⁻⁵-5·10⁻⁵-0·5·10⁻⁵; peptide 1 in fraction C, >50·16-15·11·3-4·6-2·7·9·8-8·6·3-6·2-6·2-8·8·2-10·1·4·7-2·4·1·3·6-3·1·5-0·5≤3; peptide 2 in fraction C, 0·6·3·4·8·4·3·4·0·7·1·1·9·6·3·1·0·3·7·5·0·5·1·8·1·2·9·8·1·7·5·3·6·5·0·5·1·1·0·5·0·5≤2·2·3; Mass spectra were recorded in the range from m/z range of 150-300 at a resolution of 4000 (10% valley definition). Multiply charged ions were observed corresponding to (M + 2H)²⁺, (M + 3H)³⁺, and (M + 4H)⁴⁺ protonated molecules (see Fig. 5). The help with the mass spectrometry of Dr. William Griffiths, Protein Analysis Center, Karolinska institutet, Sweden, is acknowledged. Prior to the mass spectrometry, the fractions from the HPLC had first been dried in a Speed-Vac and then peptides were re-dissolved in 10 µl of 50% methanol.

RESULTS

Reactivity of Dinitrohalobenzenes and Analogues with TrxR and Production of Superoxide—Induction of an NADPH oxidase activity in mammalian TrxR upon alkylation with DNBC could be an effect due to the alkylation of the enzyme per se, due to specific properties of DNBC, or due to a combination of these factors. To analyze this, we compared the effects on mammalian TrxR of different DNBC analogues and of alkylating agents other than dinitrophenylalkylated TrxR. The effects of alkylating agents IAA or 4-VP did not induce any NADPH oxidase activity, although TrxR was irreversibly inhibited, showing that only alkylation of the reduced enzyme was sufficient to induce an NADPH oxidase activity (Table I). The DNBC analogues 3,4-DCNB, 2,5-DCNB, 4-chloronitrobenzene, and 1,4-dichlorobenzene neither inhibited TrxR nor induced an NADPH oxidase activity, whereas a second dinitrohalobenzene compound, DNFB, both irreversibly inhibited TrxR and induced an NADPH oxidase activity (Table I). The results indicated that specific properties of dinitrohalobenzenes were needed to concomitantly alkylate and induce an NADPH oxidase activity, although TrxR was irreversibly inhibited, showing that only alkylation of the reduced enzyme was sufficient to induce an NADPH oxidase activity (Table I). The DNBC analogues 3,4-DCNB, 2,5-DCNB, 4-chloronitrobenzene, and 1,4-dichlorobenzene neither inhibited TrxR nor induced an NADPH oxidase activity, whereas a second dinitrohalobenzene compound, DNFB, both irreversibly inhibited TrxR and induced an NADPH oxidase activity (Table I). The results indicated that specific properties of dinitrohalobenzenes were needed to concomitantly alkylate and induce an NADPH oxidase activity, although TrxR was irreversibly inhibited, showing that only alkylation of the reduced enzyme was sufficient to induce an NADPH oxidase activity (Table I). The DNBC analogues 3,4-DCNB, 2,5-DCNB, 4-chloronitrobenzene, and 1,4-dichlorobenzene neither inhibited TrxR nor induced an NADPH oxidase activity, whereas a second dinitrohalobenzene compound, DNFB, both irreversibly inhibited TrxR and induced an NADPH oxidase activity (Table I). The results indicated that specific properties of dinitrohalobenzenes were needed to concomitantly alkylate and induce an NADPH oxidase activity, although TrxR was irreversibly inhibited, showing that only alkylation of the reduced enzyme was sufficient to induce an NADPH oxidase activity (Table I).
**TABLE I** Interaction of some alkylating compounds or DNCB analogues with mammalian TrxR

| Compound          | Induction of NADPH oxidase activity | Inhibition of normal TrxR activity | Irreversible inhibition |
|-------------------|-------------------------------------|-----------------------------------|--------------------------|
| Dinitrohalobenzenes | +                                   | +                                 | +                        |
| DNCB              | +                                   | +                                 | +                        |
| DNFB              | +                                   | +                                 | +                        |
| Other alkylating agents | -                                | +                                 | +                        |
| IAA               |                                    |                                   |                          |
| 4-Vinylpyridine   |                                    |                                   |                          |
| DNCB analogues    |                                    |                                   |                          |
| 1,4-Dichlorobenzene | -                                 | -                                 | NA                       |
| 1-Chloro-4-nitrobenzene | -                               | -                                 | NA                       |
| 3,4-DCNB          |                                    |                                   | NA                       |
| 2,5-DCNB          |                                    |                                   | NA                       |

**FIG. 2** Induction of NADPH oxidase activity in TrxR simultaneously incubated with DNCB and 4-VP. TrxR (0.2 μM) was preincubated 5 min at 20 °C in 50 mM Tris, 2 mM EDTA, pH 8.0, with 500 μM NADPH. Then 4-VP and DNCB were simultaneously added at concentrations given in the figure (total EtOH concentration 1% in all cuvettes). The resulting NADPH oxidase activity between 30 and 60 min after addition of the compounds was then determined by the decrease of absorbance at 340 nm.

**TABLE II** NADPH oxidation and production of superoxide by TrxR alkylated with DNCB

| Additions to cuvette | Adrenochrome formation | NADPH oxidation |
|---------------------|------------------------|------------------|
| None                | 10.9                   | 5.5              |
| +1.5 units of SOD   | 5.8                    | 8.2              |
| + Additional 1.5 units of SOD | 1.9             | 10.9             |
| + Additional 1.5 units of SOD | 0.5                 | 12.2             |
| + Additional 1.5 units of SOD | 0.3                 | 12.1             |

* Determined by increase of absorbance at 480 nm using an extinction coefficient of 4020 M⁻¹ cm⁻¹.
* Determined by decrease of absorbance at 340 nm using an extinction coefficient of 6200 M⁻¹ cm⁻¹.

**FIG. 3** Influence of DNCB in solution upon the NADPH oxidase activity of TrxR alkylated by DNCB. TrxR (0.5 μM) was preincubated 15 min at 20 °C in 50 mM Tris, 2 mM EDTA, pH 8.0, with 300 μM NADPH. Then 100 μM DNCB was added, and the incubation was continued for 20 min to allow for total alkylation. DNCB was then removed from the preincubation mixture by application to a Sephadex G-25 column from which TrxR was eluted. The eluate was divided into six samples that were simultaneously analyzed for NADPH oxidase activity with 200 μM NADPH in 50 mM Tris, 2 mM EDTA, pH 8.0, upon addition of DNCB at concentrations indicated in the figure. The reference cuvette contained 50 mM Tris, 2 mM EDTA, pH 8.0, 200 μM NADPH, and 1 mM DNCB. The curve is a Michaeli-Menten plot fitted to the experimental data, with Kₘ = 207 μM, kₜₕ = 17 min⁻¹, and an offset of 4.5 min⁻¹. The offset corresponds to the NADPH oxidase activity of the DNCB-alkylated TrxR.
formed. The NADPH oxidation also increased when SOD was added, to twice the basal level when SOD had been added in amounts sufficient enough to dismute close to all of the superoxide formed, as indicated by the total inhibition of formation of adrenochrome (Table II). If hydrogen peroxide formed by the action of SOD or the decrease in levels of superoxide was the reason for the increased NADPH oxidation upon SOD addition is not clear and has to be investigated further.

Identification of Alkylated Residues—To determine the site(s) of alkylation, DNB was in the presence of NADPH added to native TrxR at a 100-fold molar excess for 20 min at room temperature, i.e. conditions that yield a fully inactivated enzyme but concentrations and time of incubation sufficiently low to avoid extensive nonspecific alkylation. The dnp-alkylated protein was then desalted, digested with lysine-specific endoproteinase, and peptides were separated using reverse phase HPLC. Three fractions were found to exhibit absorbance at 340 nm, indicating the possible presence of dnp-alkylated peptides (Fig. A, fractions A, B, and C). These fractions were subjected to Edman degradation, where fraction A gave no amino acid sequence, fraction B contained a peptide corresponding to the carboxyl-terminal end of the enzyme, and fraction C contained two peptides from the interface domain of the enzyme (Table III). When detection of phenylthiohydantoin-derived amino acids was changed to 340 nm at the Edman degradation of the peptide in fraction B, a peak of decreasing intensity was seen during the first 2–3 cycles (not shown), which indicated that the dnp group is lost from the alkylated amino acid(s) during Edman degradation, as has been suggested elsewhere in experiments with glutathione S-transferase (33). Therefore Edman degradation could not be utilized to positively identify the residue(s) alkylated with a dnp group.

To determine residues alkylated in the peptides, we therefore utilized mass spectrometric analysis. The spectra of the analysis are given in Fig. 5 and summarized in Table IV. We found that fraction A, which gave no amino acid sequence, lacked higher molecular weight compounds that could correspond to alkylated peptides (Fig. 5A). However, the mass of compounds present in the two other fractions correlated well with the theoretical mass of dnp-alkylated peptide derivatives.

Fraction B contained the dnp-alkylated carboxyl-terminal peptide (Table III) and mass spectrometry (Fig. 5B) revealed the presence of three high molecular weight compounds (1397, 1644, and 3003 mass units, respectively). It should be noted that among the amino acid residues present in the carboxy-terminal peptide, only the Cys or Sec residues could theoretically be alkylated by DNB. The mass of none of the three compounds in the fraction correlated to a native form carboxy-terminal peptide with only one dnp group attached. Instead, the compound with a mass of 1644 agreed well with the theoretical mass of the peptide alkylated at both the Cys and the Sec residues. The 1397 compound probably represented the product of a loss of Se(dnp) from the peptide with formation of dehydroalanine at the position of Sec. The 3003 compound could be proposed to represent a dimeric compound, linked by an oxygen bridge with the Sec residues in the form of selenenic acid. The structures of these proposed derivatives of the carboxy-terminal peptide are shown in Fig. 6. The 1397 and 3003 compounds were most likely formed at drying of the sample or due to the process of mass spectrometry, while the doubly alkylated peptide should be the species originally present in fraction B at the stage of the HPLC separation.

Mass spectrometry of fraction C (Fig. 5C) showed that the peptide corresponding to the start of the interface region (peptide 1, Table III) was nonalkylated with its mass of 2690 found as one of the compounds present in the fraction. The other...
**Fig. 5.** Mass spectrometric analysis of fractions "A," "B," and "C" from the HPLC separation shown in Fig. 4. Electrospray mass spectra were recorded as described under “Experimental Procedures.” Shown are the original spectra with the molecular weights of major singly charged ions given in parentheses and sodium adducts marked with asterisks. In the insets enlargements of relevant areas are given, with calculations of the parent compound molecular weights. A shows the analysis of fraction A from Fig. 4, B of fraction B, and C of fraction C. A summary of the analysis with an interpretation of compounds present is given in Table IV.
The amino acid sequence of the native peptide representing the carboxyl-terminal end of bovine TrxR is shown in Table III and the text. \( R \) denotes the 10 first amino acids of the peptide.

Peptide was not present in its nonalkylated form with a mass of 2575.3, but had been alkylated with one dnp group, representing the compound with a mass of 2744 (Table IV).

**DISCUSSION**

The NADPH oxidase activity of native TrxR was with 200 \( \mu \)M NADPH determined to be about 0.15 min \(^{-1} \) (31), which is quite low compared with other flavoproteins (34). The increase of NADPH oxidase activity in mammalian TrxR by DNCB was shown here to be dependent not solely on the alkylation but on a combination of this with a certain property of the dinitrohalobenzenes, no doubt carried by the reactive nitro groups of these compounds.

The induced NADPH oxidase activity resembles that of GR incubated with TNBS studied in detail by Carlberg and Mannervik (35). This interaction was, however, reversible and was fully dependent on TNBS in solution. The kinetics of the TNBS induced NADPH oxidase activity in GR was most complex, sigmoidal in the absence and nonsigmoidal in the presence of NADP\(^{+} \), with the latter supporting one-electron transfer reactions (35). However, with TrxR alkylated by DNBC, addition of NADP\(^{+} \) did not change the velocity of NADPH oxidase activity (not shown) and the effects of DNBC in solution added to DNBC-alkylated TrxR (Fig. 5) did not show the complex kinetics that was found in the interaction between GR and TNBS.

Lipoamide dehydrogenase, which like GR is structurally related to mammalian TrxR (26), is known to carry diaphorase activity, *i.e.* having the capacity to transfer electrons to artificial acceptors such as dichlorophenoldindophenol or ferricyanide (reviewed in Ref. 1). It is clear that the FAD of lipoamide dehydrogenase catalyzes this diaphorase activity directly without participation of the redox active disulfide, since both lipoamide dehydrogenase treated with \(-\)SH alkylators (36) point mutated lipoamide dehydrogenase with serines instead of cysteines at the redox active disulfide (37), or other chemical modifications inhibiting the normal redox reactions (1) can leave the electron transfer to alternate acceptors unaffected or even greatly increased.

The NAD(P)H-dependent aerobic redox cycling of aromatic nitro compounds with flavoenzymes under production of superoxide is a known phenomenon (36, 38–40). The overall mechanism is believed to be reduction of the flavin by the pyridine nucleotide, followed by one-electron transfers from the flavin to the nitro groups under production of nitro anion radicals, that in turn react with molecular oxygen under production of superoxide. We believe that this is the basic mechanism for the NADPH oxidase activity in mammalian TrxR induced by dinitrohalobenzenes and that the reacting nitro groups are either in solution or, alternatively, those positioned close to the active site by a dinitrobenzene alky group of the alkylated enzyme.

We found that three residues were dnp-alkylated in TrxR incubated with TNBS treated with DNBC. The fraction with the highest absorbance at 340 nm (fraction B, Fig. 4) contained the peptide corresponding to the carboxyl-terminal end of the enzyme (see Table III and Fig. 1) with both the Cys and the Sec alkylated. In addition, we found a peptide corresponding to a part of the interface region being alkylated with one dnp group, either at the His proposed to act as a base at the active site in GR and TrxR-like enzymes (1, 26) or at its neighboring Cys...
(peptide 2, fraction C, Table III). Alkylation at all three of these residues can in theory easily explain the inactivation of the normal enzymatic activity of the enzyme, all residues being envisioned to take part at the active site of the enzyme. It is not at this stage certain which of the nitro groups of the provided dnp groups that would give rise to superoxide formation. However, we may propose the following events. First, reduction of the enzyme with NADPH, via the FAD prosthetic group and probably the NH₂-terminal GR-like redox active disulfide, must make the Cys and Sec at the carboxyl terminus, or possibly the whole active site, more accessible for alkylation, since oxidized TrxR is resistant to alkylation by DNBC (31) as well as digestion with carboxypeptidase Y (26). A combination of conformational change or reduction of a bridge between the carboxyl-terminal Cys and Sec residues² could be proposed to account for the change in accessibility for alkylation. We then propose that the FAD of the dnp-alkylated enzyme still can be reduced by NADPH but instead of two-electron transfer of reducing equivalents to the NH₂-terminal active site disulfide, as in the normal case, a nitro group of the dnp group is reduced in a one-electron transfer to form an anion radical, which in turn reacts with oxygen to form superoxide. A second consecutive step of a one-electron transfer to form another nitro radical with subsequent reaction with oxygen under formation of superoxide would regenerate fully oxidized FAD of the enzyme and NO₂ groups in the participating dnp group. Adrenochrome formation is a good measure of superoxide formation formed by univalent reduction of oxygen by reduced flavins (32). The fact that 2 mol of adrenochrome were formed per mol of NADPH by the DNBC-alkylated TrxR and that SOD completely blocked this formation (Table II) supports the notion that two superoxides are formed per NADPH (or FADH₂). Free DNBC in solution is, in addition, proposed to be able to react with the FAD prosthetic group of the alkylated TrxR, to form nitroradicals and superoxide. This is supported by the Michaeli-Menten type of kinetics of the increase in NADPH oxidase activity dependent on free DNBC added to already alkylated TrxR (Fig. 3).

The cycle of events proposed above would explain the inactivation of TrxR with DNBC and concomitant induction of an NADPH oxidase activity. Since only three residues of the enzyme were found to be alkylated, the importance of these amino acids for the catalytic activity is illustrated. It should be pointed out that the active site structure of mammalian TrxR most likely is composed of the combination of the GR-like NH₂-terminal redox active disulfide motif of one subunit and the carboxyl-terminal selenocysteine containing redox active motif of the other subunit (26), in analogy with mercuric reductase (1, 41). This implies that the nitro groups of the dnp groups at the carboxyl-terminal motif could be proposed to become positioned in the vicinity of the NH₂-terminal redox active motif, which normally contains the cysteine residues that accept reducing equivalents from FADH₂.

In this study we could not detect dnp derivatization of the NH₂-terminal GR-like active site dithiol/disulfide motif, which we previously found to be alkylated by 4-VP in a similar experiment with NADPH-dependent alkylation and identification of redox active residues of the enzyme (26). The explanation could be that, specifically, the nitro groups of DNBC sterically hinders alkylation of the NH₂-terminal redox active motif, or, more likely, that the carboxyl-terminal motif generally is more accessible and easily alkylated. It should be noted that the conditions for alkylation in this study were milder than in that with 4-VP (26).

²A redox active selenyl sulfide between the carboxyl-terminal Cys and Sec residues was recently demonstrated (L. Zhong, E. S. J. Arner, and A. Holmgren, manuscript in preparation).

It is of interest to note that the Cys as well as the Sec residue of the carboxyl-terminal peptide had been alkylated. The selenogroup of Sec usually has a high nucleophilic reactivity and a low pKa value, which makes this residue a natural target for alkylation with electrophilic compounds, providing that it is sterically accessible. The finding that the neighboring Cys residue was alkylated as well indicates that in mammalian TrxR, its sulfhydryl group has an unusual reactivity indicating a low pKa value.

Does the specific and high reactivity with mammalian TrxR of dinitrohalobenzenes like DNBC play a role in the mechanism of the immunomodulating properties of these compounds? In this context, it is of importance to note that all of the DNBC analogues that failed to inhibit TrxR or to induce any NADPH oxidase activity (Table I) previously had been tested in vivo for induction of hypersensitivity reactions and shown to provoke no reaction (30). In the same study, O₂ utilization, H₂O₂ production, and NADPH consumption in skin or liver microsomes was also measured upon addition of dinitrohalobenzenes or the DNBC analogues. All of these properties correlated well to mouse ear swelling upon application of the compounds, whereas changes in levels of GSH or GSSG did not (30). The enzyme(s) responsible for the NADPH consumption and superoxide (or H₂O₂) production were not identified, but based upon our study it is safe to conclude that TrxR is a strong candidate. How would the interaction with TrxR by dinitrohalobenzenes take part in the mechanism of immunostimulation by these compounds? Two mechanisms would be the most probable. First, Trx plays a central role in redox regulation of cell function (3, 5, 42–44), and an irreversible inhibition of TrxR would therefore with certainty affect Trx-related functions in the immune system. Second, the induced NADPH oxidase activity and increased production of superoxide (and H₂O₂ in the presence of SOD) by TrxR alkylated by dinitrohalobenzenes in combination with inhibition of the many anti-oxidant functions of the mammalian Trx system should give rise to a significant oxidative stress, which in itself is immunostimulatory (45).
Dinitrohalobenzene Reactivity with Thioredoxin Reductase

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