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On the interaction between glyceraldehyde-3-phosphate dehydrogenase and airborne particles: Evidence for electrophilic species

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Abstract

Many of the adverse health effects of airborne particulate matter (PM) have been attributed to the chemical properties of some of the large number of chemical species present in PM. Some PM component chemicals are capable of generating reactive oxygen species and eliciting a state of oxidative stress. In addition, however, PM can contain chemical species that elicit their effects through covalent bond formation with nucleophilic functions in the cell. In this manuscript, we report the presence of constituents with electrophilic properties in ambient and diesel exhaust particles, demonstrated by their ability to inhibit the thiol enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is irreversibly inactivated by electrophiles under anaerobic conditions by covalent bond formation. This inactivation can be blocked by the prior addition of a high concentration of dithiothreitol (DTT) as an alternate nucleophile. Addition of DTT after the reaction between the electrophile and GAPDH, however, does not reverse the inactivation. This property has been utilized to

Abbreviations: PM, particulate matter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; GSSG, oxidized glutathione; GSH, reduced glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; DEP, diesel exhaust particles; NAD^+, oxidized nicotinamide adenine dinucleotide; G-3-P, glyceraldehyde-3-phosphate; H_2O_2, hydrogen peroxide; NEM, *N*-ethylmaleimide; DMNQ, 2, 3-dimethoxy-1, 4-naphthoquinone; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethylsulfoxide; NADH, reduced nicotinamide adenine dinucleotide

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develop a procedure that provides a quantitative measure of electrophiles present in samples of ambient particles collected in the Los Angeles Basin and in diesel exhaust particles. The toxicity of electrophiles is the result of irreversible changes in biological molecules; recovery is dependent on resynthesis. If the resynthesis is slow, the irreversible effects can be cumulative and manifest themselves after chronic exposure to low levels of electrophiles.

Keywords: Electrophile; Glyceraldehyde-3-phosphate dehydrogenase; Airborne particulate matter; Diesel exhaust particles; Ultrafine particles

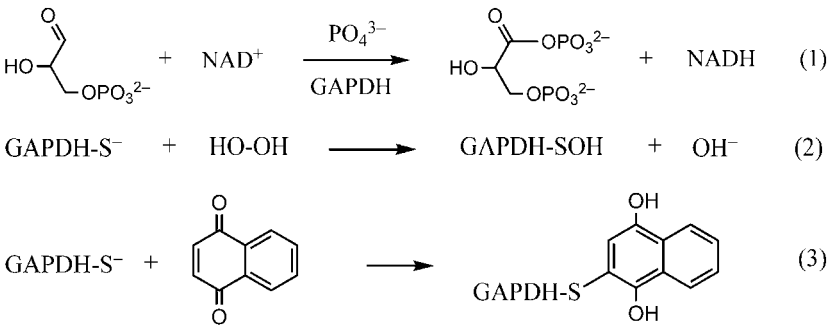
1. Introduction

A commonly proposed basis for the adverse health effects of air pollution is oxidative stress induction, mediated by chemically reactive species present in both particulate and volatile components (Baeza-Squiban et al., 1999; Frampton et al., 1999; Li et al., 2000, 2002; Donaldson et al., 2001; Squadrito et al., 2001; Nel, 2005). In a state of oxidative stress, the ratio of oxidized to reduced forms of key cellular antioxidants such as glutathione, is significantly increased (see for example, Schafer and Buettner, 2001). The ratio of oxidized form (GSSG) to the reduced form (GSH) of this thiol containing compound can be altered in two ways, by increasing the concentration of GSSG or decreasing the concentration of GSH. GSSG is the product of the reaction of GSH with hydrogen peroxide, which is generated in the cell through an electron transfer reaction between a source such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen, with disproportionation of the initial product, superoxide. Particulate and vapor phase constituents of ambient air contain compounds capable of this reaction which has been attributed to the induction of oxidative stress by air pollution (Squadrito et al., 2001; Donaldson et al., 2003; Li et al., 2004). An alternative mechanism for the induction of oxidative stress, however, is the depletion of GSH by conjugation with an electrophilic species present in the pollutant. Electrophilic species could furthermore form covalent bonds with nucleophilic centers such as thiolates in cellular proteins, disrupting cellular functions and leading to toxicity.

Covalent bond formation between reactive environmental chemicals and biological molecules is important because of its irreversible nature. Depending on the turnover rate of the altered biological molecule, chronic exposure could lead to a progressive increase in levels of irreversibly altered proteins. The cellular changes resulting from such a process would be quite different from an acute exposure to a reactive oxygen species (ROS) generator, since at low concentrations, the effects of thiol oxidation by ROS could be reversed by the multiple cellular antioxidant processes. If compounds capable of this irreversible action were present in ambient air, they could be important contributors to air pollution toxicity. For this reason, we have been considering approaches to assess, in quantitative terms, the electrophilic properties of air pollutants. As the concentration of electrophiles in air is expected to be low, we selected an enzyme to use as a nucleophile, reasoning that enzyme inhibition by a small quantity of electrophile would be amplified by changes in catalytic activity. Under conditions of fixed enzyme, substrate and cofactor concentrations, the changes in enzyme activity would be proportional to the concentration of the electrophilic inhibitor. By keeping the enzyme concentration in the assay small, the quantity of inhibitor needed to reduce activity would also be small, and changes in enzyme activity due to inhibitor could be easily monitored over time.

In this manuscript, we report the development of a quantitative analytical procedure to detect electrophiles that can be applied to the study of air pollution samples, using the active site of the thiol enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) as the target nucleophile. Reactions (2) and (3) provide the underlying basis for the use of GAPDH as a nucleophile based enzyme. The enzyme has a well-established mechanism involving a nucleophilic thiol and its activity can be monitored by NADH formation (reaction (1)). It is inhibited by electrophiles (reaction (3)), but it may also be inhibited by ROS (reaction (2)). In the study, possible confounding effects of ROS were assessed first, and the results indicated that all sources of ROS would have to be eliminated to prevent direct and indirect thiol oxidation. Then, the effects of the electrophile, *N*-ethylmaleimide on

the enzyme were examined, followed by studies examining GAPDH inhibition by diesel and ambient PM preparations. These results were used to develop a general method for electrophile content in samples. To meet the objective of a high throughput, reproducible assay, an anaerobic procedure using chicken muscle GAPDH was developed. The assay was then applied to diesel exhaust particles (DEP) and aqueous suspensions of ambient air particles.



2. Methods

Sections 2.1–2.6 describe general procedures used for the studies. Section 2.7 describes the assay protocols used for the test samples.

2.1. Materials

Chicken muscle GAPDH, dithiothreitol (DTT), oxidized nicotinamide adenine dinucleotide (NAD^+), glyceraldehyde-3-phosphate (G-3-P) and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. (St. Louis, MO), *N*-ethylmaleimide (NEM) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) was purchased from Calbiochem (San Diego, CA). Ethylenediamine tetraacetic acid (EDTA) was purchased from Fisher Scientific Co. (Los Angeles, CA). Sodium phosphate buffer was treated with chelex resin (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions to remove metal contamination.

2.2. Particle samples

2.2.1. Diesel exhaust samples

The DEP samples were obtained from the Department of Environmental Sciences of the National Institute of Environmental Studies, Tsukuba, Japan. They were generated by a light duty diesel engine and collected on glass filters by procedures described earlier by Sagai et al. (1993). The extracts were prepared by suspension in dichloromethane and ultrasonication at room temperature for 20 min, followed by centrifugation. The supernatant was evaporated and reconstituted in dimethylsulfoxide (DMSO) at concentrations required. Aliquots of this solution were added to the GAPDH reaction mixture to attain concentrations between 10 and 100 $\mu g\,mL^{-1}$. The final DMSO concentration was 1%.

2.2.2. Ambient particulate matter (PM) samples

These samples were obtained by means of a particle concentrator—BioSampler tandem system described by Kim et al. (2001a, b) situated at Riverside, CA. Sampling was conducted in the summer and fall of 2006. Highly concentrated liquid particle suspensions were obtained by connecting the concentrated output flow from the concentrator to a liquid impinger (BioSampler^{TM}, SKC West Inc., Fullerton, CA). Particles are injected into the BioSampler^{TM} in a swirling flow pattern so that they can be collected in a small volume of water by a combination of inertial and centrifugal forces. Samples from this collection system were used as the suspension itself without further treatment.

Ultrafine particle suspensions obtained from the BioSampler were collected at an outdoor site in Riverside, at the eastern end of Los Angeles Basin. The quantity of particles was limited by the concentration of each suspension. Because of their dilute nature, the maximum volume allowable in the procedure, 0.1 mL, was used. The particle concentrations in each sample are shown in Table 1.

Table 1
Electrophiles in ambient air samples

Sample date of collection, Riverside	% inhibition/ aliquot[a]	PM concentration ($\mu g\,mL^{-1}$)	% inhibition μg^{-1}[b]	Volume of air sampled (m^3)	% inhibition m^{-3} [c]	NEM equivalent inhibition[d]
31 August	9.26±1.71	133.3	0.695±0.128	4.8	144±26.7	25.5±4.71
14 September	22.3±1.62	32.08	6.96±0.506	4.8	893±65.0	255±18.6
21 September	12.7±2.45	41.44	3.07±0.590	4.4	483±92.8	113±21.6
28 September	18.0±2.00	103.2	1.74±0.194	5.5	310±34.5	63.9±7.10
5 October	8.83±0.603	34.82	2.53±0.173	4.2	471±32.2	92.9±6.35
12 October	8.94±1.20	19.81	4.51±0.604	5.0	559±74.9	165±22.2

Electrophile-dependent inhibition was estimated by subtracting electrophile-independent inhibition (pre DTT) from total inhibition (no Q1 DTT).
Data are shown as % inhibition compared with control (mean±S.E., $n = 3$).
[a]Values were normalized to volume (100 μL) of aliquot added to incubation.
[b]Values were normalized to volume (100 μL) of aliquot added to mass of particles.
[c]Values were normalized to volume (100 μL) of aliquot added to equivalent of original air volume.
[d]% of NEM (1 ng) equivalent inhibition/mass (μg).

2.3. Reaction of test sample/compound with GAPDH

GAPDH inhibition assays consist of two steps, enzyme inactivation by the test material and the determination of remaining active enzyme. The reaction between enzyme and test material was initially conducted under aerobic and anaerobic conditions, described below, to determine oxygen-dependent and oxygen-independent reactions. Inactivation reactions are allowed to proceed for a defined time period and aliquots of the reaction mixture removed, quenched with DTT and transferred to ice. Chilled samples are then assayed for enzyme activity, described in Section 2.6.

2.4. Aerobic (oxygen-dependent) reaction conditions

The incubation mixture (0.5 mL) consisted of 80 mM sodium phosphate buffer, pH 7.4, 5 unit mL^{-1} GAPDH, 1 mM EDTA, and test compound in glass tubes (16 × 100 mm) open to the atmosphere. After incubation at 25 °C for time periods described in each experiment, 0.5 mL of cold DTT solution (2 mM in 0.1 M sodium phosphate buffer, pH 7.4) was added and the tubes immediately placed in ice.

2.5. Anaerobic (oxygen-independent) reaction conditions

Anaerobic incubations (total volume of 0.5 mL) were performed in glass test tubes (16 × 100 mm^2) sealed with rubber septa with continuous moisturized-argon gas flow using needles as inlets and outlets. Prior to the experiment, all solutions were deoxygenated in sealed vials with moisturized-argon gas flow. The reaction was started by addition of the GAPDH solution through the rubber septum using a gastight syringe. Incubation was performed in a 25 °C water bath with shaking (75±5 min^{-1}) for the times indicated in figure legends. Argon inside the tubes was maintained under positive pressure during the incubation to prevent oxygen contamination. The reactions were stopped by adding 0.5 mL of cold DTT solution (2 mM in 0.1 M phosphate buffer, pH 7.4) with a gastight syringe to each tube and the tubes placed on ice.

2.6. GAPDH activity

Aliquots (0.2 mL) of the incubation mixtures above were assayed by the method of Dagher and Deal (1982). The assay mixture (1 mL) consisted of 0.5 units of GAPDH, 1 mM NAD^+, 1.5 mM G-3-P, 86 mM sodium phosphate buffer, pH 7.4, 80 μM EDTA and 1 mM DTT. The mixture without G-3-P was incubated at 25 °C for 2 min and the reaction started by adding the G-3-P solution and mixing by repeat pipetting. Absorbance at 340 nm was then monitored for 40 s and linear range of the increase, usually between 10 and 20 s, was used. Reduced nicotinamide adenine dinucleotide (NADH) production was estimated by its extinction coefficient of 6290 $M^{-1}\,cm^{-1}$.

2.7. Analytical assay protocol for application to environmental samples

Based on a set of initial experiments, a protocol for an analytical assay that can be applied to test samples was developed. The assay is performed under anaerobic conditions in the presence and absence of DTT. Two identical sets of analysis tubes are prepared. In set 1, GAPDH is incubated with the test samples or standard (NEM, 0.5 μM) under anaerobic conditions (described in Section 2.5) for 120 min in the absence of DTT. The enzyme activity found in this set represents total inhibition, including both thiol-mediated (electrophilic inhibition) and non-thiol-mediated. At the end of the incubation period, 1 mM DTT is added to prevent further electrophilic reaction and oxidation caused by redox compounds in the sample, so that GAPDH activity can be assayed under aerobic conditions.

Set 2 includes 1 mM DTT and the incubation is otherwise identical to the first set. This set of samples is used to determine non-thiol-mediated inhibition. The electrophile-mediated inhibition is estimated by subtracting the inhibition in set 2 (non-thiol-mediated) from inhibition in set 1 (total inhibition).

2.7.1. Analytical assay details

Enzyme reaction solutions are prepared as follows: set 1: GAPDH ($6.25\,\text{unit}\,\text{mL}^{-1}$), and 1.25 mM EDTA in 0.1 M phosphate buffer, pH 7.4 and set 2: GAPDH ($6.25\,\text{unit}\,\text{mL}^{-1}$), and 12.5 mM EDTA in 0.1 M phosphate buffer, pH 7.4, with 1.25 mM DTT. The solutions are prepared in 25-mL pear-shaped flasks which are sealed with rubber septa and deoxygenated for >45 min in ice by argon gas flow to the flask headspace from inlet to outlet needles. Samples are prepared in glass tubes (16×100 mm) with rubber septa. DDW and NEM (2.5 μM) are included as controls. PM samples are subjected to ultrasonication for 1 min prior to use to break coagulated particles. Tubes are deoxygenated by argon gas flow into headspace from inlet to outlet needles for 15–20 min. After deoxygenation, argon inside the pear-shaped flask and test tubes is maintained under positive pressure to prevent oxygen contamination during the anaerobic process. The reaction is started by anaerobically transferring 0.4 mL of GAPDH solution (set 1 or 2) to the two sets of sample test tubes using a gastight syringe. The mixtures are incubated for 120 min at 25 °C with shaking ($75 \pm 5\,\text{min}^{-1}$). Reactions are quenched by adding cold DTT solution (0.5 mL, 2 mM) to the test tubes with a gastight syringe and the tubes placed in ice. Aliquots of the samples are assayed for GAPDH activity according to the procedure described in Section 2.6.

2.7.2. Calculation of electrophile content

The electrophile content in the test samples is determined by comparing the % GAPDH activity inhibited after 120 min incubation in set 1 (without DTT) and set 2 (DTT treated) tubes. Non-thiol-dependent inhibition, i.e., that found for in set 2, is then subtracted from total inhibition, that found in set 1, for each sample. The resulting electrophile based inhibition is normalized to mass (μg) or air sample volume. GAPDH inhibition equivalent to 1 ng NEM is also calculated from the NEM standards and the inhibition by all samples are normalized to NEM equivalents.

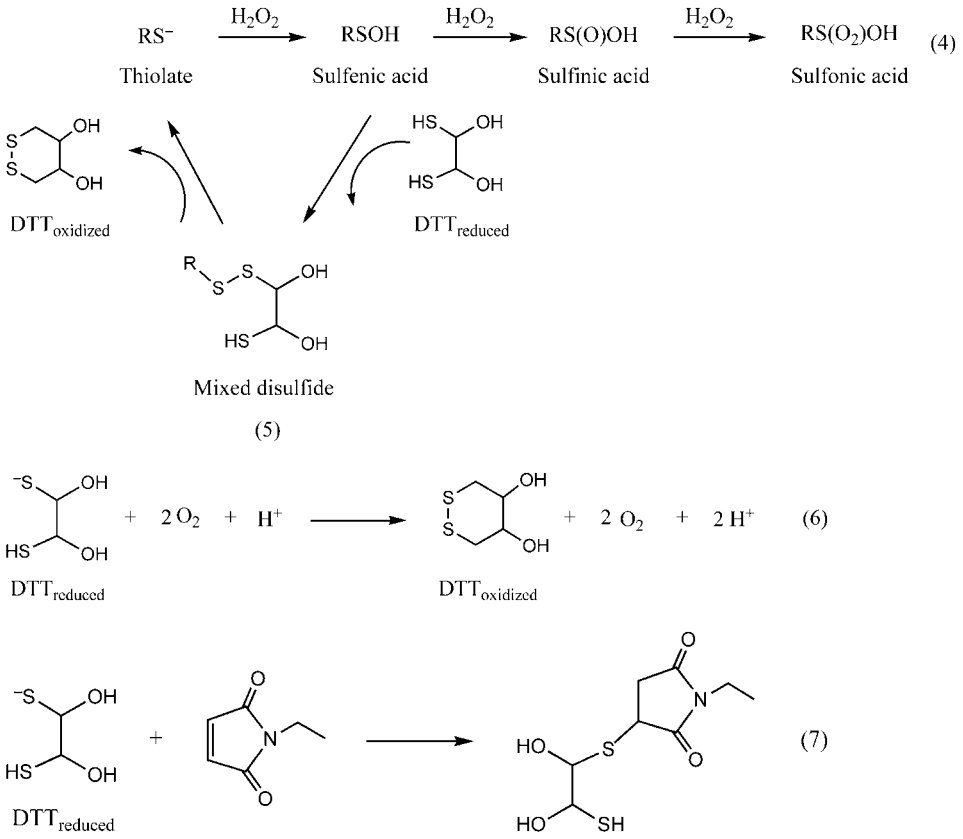
2.8. Data analysis

The linear and non-linear regression analyses were performed with GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA).

3. Results

The rationale for the assay was to utilize the dependency of GAPDH catalysis on thiol chemistry, i.e., if the thiol is modified by a covalent bond, the enzyme would be inactivated. The proportion inactivated would be dependent on the concentration of electrophile, providing there were no oxidation-based reactions occurring. The sensitivity of the assay is based on the reduction of available catalyst, which will decrease the rate of the reaction when standardized conditions are used for exposure and activity determination. The conjugate base of thiols, the thiolate, is highly reactive; it will react with hydrogen peroxide to form sulfenic acid or higher oxidation states (reaction (4)) or it can react with electrophiles such as *N*-ethyl maleimide to form covalent bonds (reaction (10)). In developing the assay, it was necessary to determine conditions under which the thiolate is oxidized by ROS such as hydrogen peroxide (H_2O_2) and conditions under which an oxidized thiol can be reduced. The dithiol, dithiothreitol (DTT) is a reactive thiol which undergoes

redox (reaction (6)) and nucleophilic (reaction (7)) reactions. It was used in both capacities in the initial experiments. Ascorbate is a reducing agent with no nucleophilic properties and was used as an electron source for H_2O_2 generation. These reagents and a redox active quinone, dimethoxy-1,4-naphthoquinone (DMNQ), were used to examine GAPDH after oxidation by air, oxidation by H_2O_2, directly and indirectly by electron transfer from ascorbate.



3.1. Enzyme preparations

Initial studies were performed with yeast GAPDH but it was found to be unstable and samples varied in their activity, so in experiments leading to the analytical procedure, the less expensive and more stable chicken GAPDH was used. The activity of chicken GAPDH was more reproducible; in several different experiments, activities of 0.5 units of chicken GAPDH were 0.783 ± 0.031 (aerobic conditions, $n = 9$) and 0.925 ± 0.058 (anaerobic conditions, $n = 5$) nmol NADH formed s^{-1}.

3.2. Autoxidation of GAPDH and its reduction by DTT

As a thiol protein, GAPDH readily undergoes autoxidation, i.e., the thiol is oxidized to the sulfenic acid or higher state at which the enzyme is inactive (reaction (4)). Consistent with this notion, time-dependent air oxidation of chicken muscle GAPDH was observed ($18.8 \pm 5.78\%$, $n = 4$, within 30 min and $53.8 \pm 11.8\%$, $n = 3$, in 120 min at 25 °C). The sulfenic acid state, also formed in a reaction with hydrogen peroxide, reacts with small thiols such as GSH to form mixed disulfides. Both sulfenic acids and mixed disulfides are reduced to thiols by DTT (reaction (5)). Thus, enzyme inactivated by oxidation to either sulfenic acid or disulfide states can be recovered by addition of DTT, used here as a reducing agent (Poole et al., 2004; Rodriguez et al., 2005). To insure that the GAPDH to be used was maximally reduced, GAPDH that had been exposed to air was reduced with DTT for varying times and concentrations to

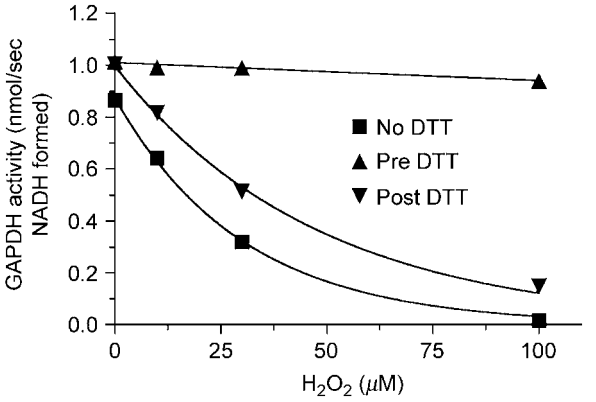


Fig. 1. Aerobic interaction between GAPDH and hydrogen peroxide (H_2O_2). GAPDH (5 unit mL^{-1}) and hydrogen peroxide, at the indicated concentrations, were incubated for 30 min at 25 °C, then GAPDH activity was assayed as described in Section 2. DTT (1 mM) was added to the reaction mixture before the hydrogen peroxide (pre DTT) or after the reaction (post DTT).

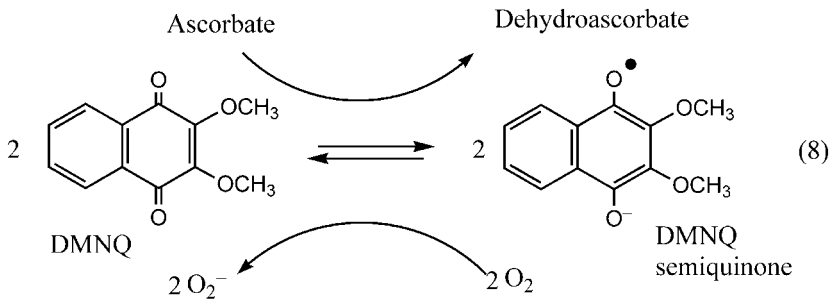
establish optimal conditions for maximal reactivation. A concentration of 1 mM was found to be optimal when incubated for 2 min at 25 °C and these conditions were used to obtain preparations with maximal enzyme activity (data not shown).

3.3. Effect of hydrogen peroxide and its reversal by DTT

As stated above, hydrogen peroxide (H_2O_2) oxidizes thiols to the sulfenic acid and higher states (Poole et al., 2004), and when the thiol function at the active site of an enzyme is oxidized, it is inactive (Poole et al., 2004; DeYulia and Carcamo, 2005). To determine conditions for H_2O_2 based GAPDH inactivation, the enzyme was incubated with H_2O_2 at concentrations up to 100 μM for 30 min. As shown in Fig. 1, the enzyme was inactivated in a concentration-dependent manner. This loss in activity was completely protected by the presence of DTT (1 mM), acting as an alternate thiol, to destroy the peroxide. However, when DTT was added after inactivation, recovery was minimal, indicating that the hydrogen peroxide-mediated thiol oxidation may be to higher oxidation states such as the sulfinic acid or sulfonic acid states which are not reversible with DTT under these conditions.

3.4. Indirect GAPDH inactivation by 2,3-dimethoxy-1,4-naphthoquinone (DMNQ)

The H_2O_2 based oxidation of GAPDH thiols can also occur by an indirect process, in which peroxide is generated by electron transfer from a reducing agent such as ascorbate to oxygen to generate superoxide and other ROS. DMNQ is a redox quinone, i.e., it has the ability to transfer electrons from ascorbate to oxygen (Gant et al., 1988) (reaction (8)). The initial product of the reaction is superoxide, which rapidly disproportionates to H_2O_2 (reaction (9)), which could then oxidize the GAPDH thiol to the inactive sulfenic acid or higher state and inactivate the enzyme. To evaluate this possibility, DMNQ at 30 μM, was incubated with GAPDH in the presence of the electron donor, ascorbic acid. Under aerobic conditions, the DMNQ/ascorbate mixture inactivated the enzyme (Fig. 2), but the mixture had no effect under anaerobic conditions (data not shown). Similar generation of H_2O_2 by DTT and quinones has been demonstrated in earlier studies (Kumagai et al., 2002).



(8)

$$2\,O_2^- + 2\,H^+ \longrightarrow O_2 + H_2O_2 \qquad (9)$$

These experiments show that GAPDH thiol(s) can be oxidized by hydrogen peroxide, introduced directly and indirectly from an electron transfer

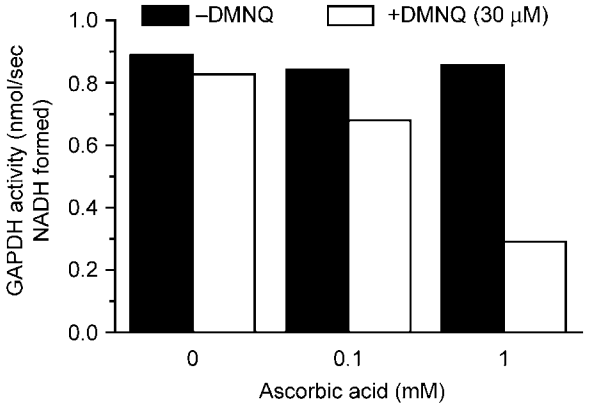


Fig. 2. Aerobic interaction between GAPDH and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). GAPDH (5 unit mL^{-1}) was incubated in the absence (closed bars) or presence (open bars) of DMNQ (30 μM) with the indicated concentrations of ascorbic acid for 30 min, then GAPDH activity assayed.

reaction between ascorbate and oxygen, catalyzed by the electron transfer agent, DMNQ. They also showed that when the oxidation is minimal, i.e., limited to the sulfenic acid state, the oxidized thiol can be reduced with DTT.

3.5. Electrophile based inhibition of GAPDH: N-ethylmaleimide (NEM)

The interaction of the electrophile, NEM, with GAPDH was examined next. This compound is used extensively as a thiol-inactivating reagent in biochemistry because of its highly electrophilic α,β-unsaturated carbonyl system (reaction (10)). Unlike quinones, NEM is devoid of redox activity under physiological conditions. Initially, the rate of inactivation was determined at 1 μM NEM over a 45 min incubation period (Fig. 3A). The activity decayed with a first-order process, with a half-life of about 9 min. The relationship between concentration and inactivation was not linear, exhibiting saturation (Fig. 3B). The data collected under these conditions were fitted to a one site binding expression to determine a concentration at which 50% inhibition would be observed. The value was 0.805 ± 0.127 μM. Under anaerobic conditions, pre-incubation with 1 mM DTT protected GAPDH activity from NEM-mediated inhibition (Fig. 6). Based on this result, NEM was used in subsequent experiments as a control electrophilic inhibitor of GAPDH.

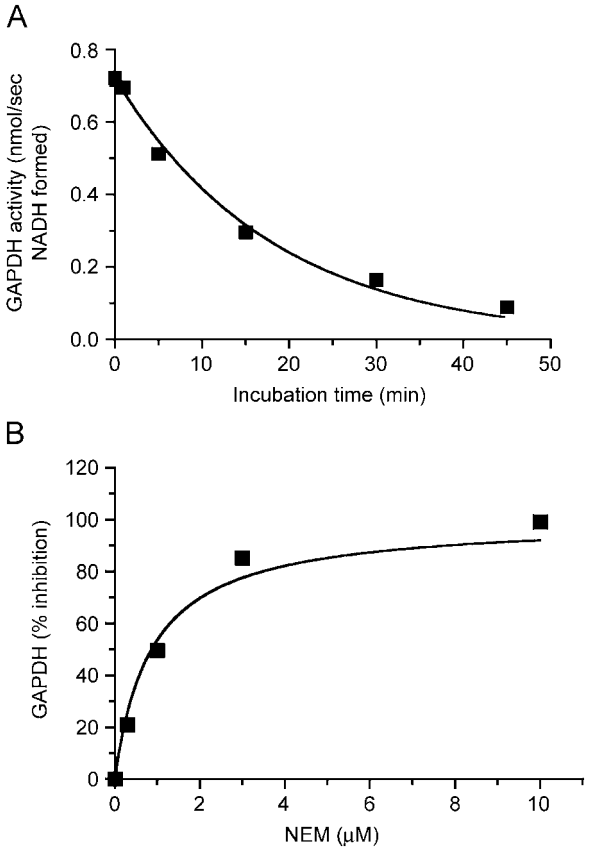
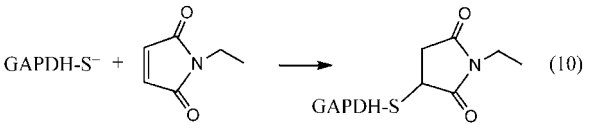


Fig. 3. Aerobic interaction between GAPDH and *N*-ethylmaleimide (NEM). (A) NEM (1 μM) and GAPDH (5 unit mL^{-1}) were incubated under aerobic conditions for the indicated times, then GAPDH activity was assayed. (B) NEM, at the indicated concentrations, was incubated with GAPDH (5 unit mL^{-1}) for 30 min under aerobic conditions after which enzyme activity was assayed as described in Section 2. The line represents the fit of the data to a one site-binding hyperbola with an apparent KD value of 0.867 ± 0.144 μM.



3.6. Effects of metals

A loss of activity, which could be reversed by the addition of DTT, was observed in non-treated controls under anaerobic conditions that varied with experiments (10–70%) (data not shown). To determine whether the loss was due to oxygen contamination or to other substances in the solution

such as metals, GAPDH inhibition under anaerobic conditions was determined in the presence of the metal chelator, ethylenediamine tetraacetic acid (EDTA), and/or DTT. EDTA prevented the loss of activity in the presence or absence of DTT (Fig. 4A), indicating that it was mostly due to metal contamination. Since the addition of EDTA (1 mM) to the incubation mixture did not significantly alter NEM-dependent GAPDH inhibition (Fig. 4B), we decided to add 1 mM EDTA in the incubation mixture to prevent metal-mediated inhibitions for the standard procedure. However, the experiment of the PM samples (Fig. 6) was performed without EDTA addition. In this experiment, corrections for the enzyme available to electrophile action were estimated from calculations using two controls, with and without DTT addition, after incubations.

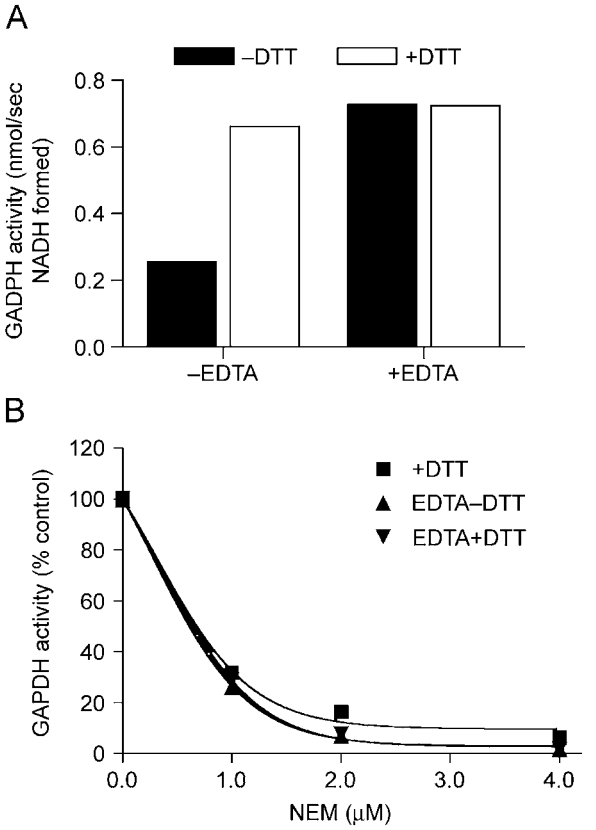


Fig. 4. Effects of metals on GADPH activity. (A) GAPDH (5 unit mL^{-1}) was incubated with or without EDTA (1 mM) at 25 °C for 120 min under anaerobic conditions. The incubations were terminated by adding cold buffer with (open bars) or without (closed bars) DTT (1 mM) and GAPDH activity measured. (B) The concentration-dependent inhibition of GAPDH by NEM was determined in the presence (triangles) or absence (squares) of EDTA (1 mM) under the same conditions as (A). The reactions were terminated by adding cold buffer with (+DTT) or without (−DTT) 1 mM DTT and GAPDH activity measured.

3.7. Assay protocol

The results of the preceding experiments were used to develop a simple, routine assay that would permit multiple sample analyses. The conditions are summarized below:

1. Incubations were conducted anaerobically, under argon to eliminate contributions from ROS-dependent reactions.
2. EDTA was included in the incubation mixtures to prevent metal based loss of activity under anaerobic conditions.
3. Enzyme activity was determined after incubation for a single time point, at 120 min. This determination assumes a log-linear rate or first-order rate of inactivation. Since the reagent concentrations, including enzyme, are arbitrarily fixed, the rate of inactivation should be dependent on the concentration of electrophile added.
4. NEM, at a concentration of 0.5 μM, was used as a standard to monitor the overall procedure from experiment to experiment.

These assay conditions limited the interpretation somewhat but allowed the determination of electrophiles, defined by their ability to inactivate GAPDH, in a given sample in quantitative terms. The activity could be expressed per mass or volume of air sampled and compared with a fixed concentration of NEM, incubated as a control for each experiment.

3.8. Application of assay

3.8.1. DEP extracts

Using the conditions above, the ability of the DEP extract to inactivate GAPDH was determined. A concentration-dependent inactivation was observed which was substantially reduced by preexposure of the extract to 1 mM DTT (Fig. 5). At a concentration of 100 μg mL^{-1}, the inactivation was not completely blocked by the DTT, but reduced the inactivation from 84% to 38%. The limitation in inhibition was also observed with NEM (Fig. 3). We do not have an explanation for the residual activity.

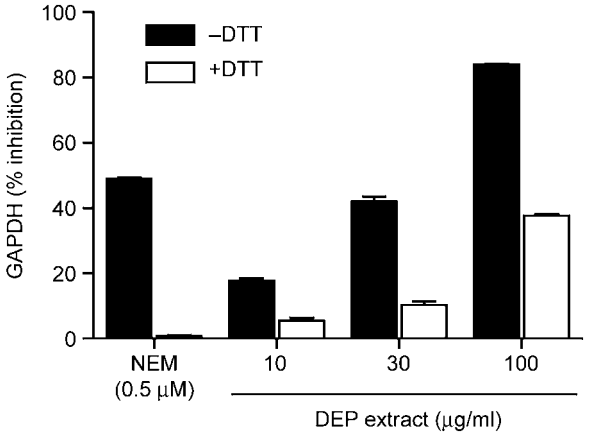


Fig. 5. Anaerobic interaction between GAPDH and DEP extract. GAPDH (5 unit mL^{-1}) and DEP extract, at 10, 30, 100 $\mu g\,mL^{-1}$, were incubated in the absence (closed bars) or presence (open bars) of 1 mM DTT at 25 °C for 120 min under anaerobic conditions. The reactions were stopped by the addition of cold DTT (final concentration of 1 mM), GAPDH activity was then assayed. The data are mean ± S.E. ($n = 3$). NEM (0.5 μM) was used as a standard.

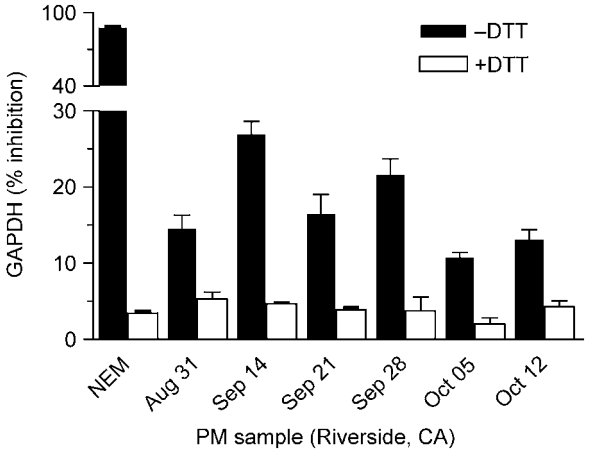


Fig. 6. Anaerobic interaction between GAPDH and ambient BioSampler collections made in Riverside, CA. GAPDH (5 unit mL^{-1}) and BioSampler suspension (0.1 mL) were incubated in the absence (closed bars) or presence (open bars) of 1 mM DTT at 25 °C for 120 min under anaerobic conditions. Reactions were stopped by the addition of cold DTT (final concentration of 1 mM), and GAPDH activity then assayed. The experiment used the largest volume (0.1 mL) possible of BioSampler suspension and the concentrations ranged from 1.98 to 13.3 $\mu g\,mL^{-1}$. The results shown express activity as % of inhibition per aliquot (0.1 mL of sample) (mean ± S.E., $n = 3$). Details of the sample analyses are summarized in Table 1. NEM (0.5 μM) was used as a standard.

3.8.2. *Ambient PM samples*

A set of ambient samples collected by the concentrator-BioSampler system at a site in Riverside, CA, was then analyzed under these standardized conditions. The samples were used as the original aqueous suspension. As the standardized conditions limited the volume of sample allowed, aliquots of 0.1 mL of the BioSampler suspensions were used, which was the largest volume permitted under the conditions of the assay. The results of the GAPDH inactivation are shown in Table 1 and Fig. 6. The results show that the samples exhibited the ability to inactivate GAPDH under anaerobic conditions by a process that was blocked by allowing the suspension to react first with DTT at 1 mM (Fig. 6). Table 1 summarizes the results and the properties of the individual samples used. Samples for 14 September and 12 October 2006 were particularly active when the results are normalized to mass or to volume of air sampled. However, inactivation per unit mass varied considerably, likely due to the varying chemical composition of PM at that site.

4. Discussion

A quantitative assay for electrophiles in air samples has been developed and applied to ambient and diesel exhaust PM. Both these types of samples were found to contain electrophiles. PM samples collected in Riverside over a period of 6 weeks during the summer months exhibited a 10-fold variation in electrophilic content as measured by our assay. The assay is based on the ability of electrophiles to react with the nucleophilic thiol in GAPDH. This thiol has a low pK_a (~6) so that at pH 7.4, most of thiol is in the ionized thiolate state that reacts with both electrophiles and hydrogen peroxide. Wide variation in the structures of electrophiles capable of GAPDH inactivation has been reported. Active compounds include acrylonitrile (Campian et al., 2002), a quinone imide (Dietze et al., 1997) and 1,4-benzoquinone (Rodriguez et al., 2005). The wide range of chemical structures that inactivate GAPDH make it a potentially useful probe for electrophilic species in ambient air samples. The advantage in using an enzyme to assay electrophiles is the catalytic property, which allows amplification of the signal by monitoring enzyme activity. However, it is possible that not all electrophiles will be detected by GAPDH, due to variability in the ability to bind the target thiol.

The assay does not identify the specific chemical species responsible for the inhibition, but provides a

metric that can be used to compare samples containing atmospheric particle and semi-volatile components for electrophile content. Electrophilic chemicals such as quinones have been detected in DEP (Schuetzle et al., 1981; Cho et al., 2004) and ambient air (Cho et al., 2004; Valavanidis et al., 2006). Additionally, aliphatic electrophiles such as acrolein and crotonaldehyde have been found in air samples collected in two California roadway tunnels (A. Eiguren-Fernandez, personal communication).

The DEP used here and their organic extracts have been used extensively in toxicological studies of PM (Hiura et al., 1999; Takizawa et al., 2000; Bonvallot et al., 2001; Koike and Kobayashi, 2005; Sugimoto et al., 2005; Inoue et al., 2006), and the results support the notion that the resulting toxicity involves oxidative stress induction. However, there is no direct evidence indicating that the oxidative stress is due only to ROS production. For example, in studies using a similar DEP sample toxicological responses were attenuated by the addition of *N*-acetylcysteine (Hiura et al., 1999; Hirano et al., 2003; Koike and Kobayashi, 2005). This protection could be due to the action of *N*-acetyl cysteine as an alternate thiol that reacts with ROS, or as an alternate nucleophile, which reacts with DEP electrophiles. The present studies demonstrate that DEP have electrophilic properties as well as the electron transfer properties demonstrated previously (Cho et al., 2005).

The current results demonstrate for the first time that extracts of DEP and aqueous suspensions of ambient PM collected in the Los Angeles Basin contain electrophilic chemical species that are capable of the anaerobic inactivation of GAPDH. The presence of electrophiles in ambient air, either in the particulate or vapor phase, has important exposure implications due to the irreversibility of the reaction between electrophiles and tissue nucleophiles. Once a covalent bond is formed between an electrophile and a nucleophilic center on a biological target, recovery from the event requires resynthesis of the target. Although the concentration of electrophiles in ambient air could be very low, if the turnover time of the target is long, inactivation due to low concentrations of electrophiles could be cumulative and over time, cause functional changes in affected cells. In contrast, cells could recover from exposure to low concentrations of ROS generating chemical species because of cellular capacity for reduction of oxidized thiols. (Claiborne et al., 1999; Poole et al., 2004).

Epidemiologic data support cumulative exposure–response relationships for ambient PM. Relationships between acute asthma outcomes and PM in panel studies with daily repeated measurements often show more robust associations with longer averaging times of the current plus several days prior to the measurement of the outcome as compared with the just the current or previous day's PM (Gielen et al., 1997; Delfino et al., 1998, 2002, 2004; Pope and Dockery, 2006). Furthermore, associations between cardiorespiratory mortality and ambient PM have been reported to be larger for averaging time scales of weeks to a few months as compared with a few days (reviewed by Pope and Dockery, 2006).

In summary, the presence of electrophilic substances has been demonstrated in samples of DEP and suspensions of ambient air particles. Based on the preliminary results, an assay was developed that would allow multiple samples to be tested under conditions that compare electrophilic properties against a NEM standard, thereby providing the ability to compared sample electrophile content in a quantitative manner. The quantitative relationship between electrophile content and toxicity has yet to be established and approaches to this problem are currently under investigation.

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