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Hydrazide derivatives produce active oxygen species as hydrazine

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Abstract

It is well documented that some hydrazines are quite sensitive to oxidation and may serve as the electron donor for the reduction of oxygen, whereas hydrazides are not believed to react directly with oxygen. Data presented in this paper show that both hydrazides and hydrazines share an N–N moiety, which is assumed to react with atmospheric oxygen and produce oxygen radicals, at various degrees of efficiency. Since spectrometric measurements of hydrazide just after solubilization showed that the molecular mass remains constant in the absence of oxygen, we can conclude that hydrazides do not react with the oxygen through a slow spontaneous hydrolytic release of hydrazine. However, hydrazine is more reactive than hydrazide, which requires hours rather than minutes to produce measurable quantities of radical species. Differences were also apparent for various substituted derivatives. The reaction was significantly enhanced by the presence of metal ions. Data reported here demonstrate that hydrazides cause irreversible damage to the prosthetic group of proteins as well as causing degradation of the polypeptide chain into small fragments.

Keywords: Hydrazine; Hydrazide; Protein degradation; Oxygen radicals

1. Introduction

Hydrazine is a hazardous chemical and its uses in the laboratory and industry range from waste water treatment, to a reducing agent in nickel plating, a chain extender in

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the polymerisation of polyurethane, a rocket propellant as well as an intermediate in industrial synthetic chemistry [1]. In agriculture it is used in tobacco cultivation and in potato and onion storage [2]. Many studies have reported that hydrazines cause a number of very diverse toxic effects including peripheral neuropathy, degeneration of sexual glands, hepatoxic effects, hyperglycemia, and cancer [3]. If necessary, steps can be taken to avoid human exposure to hydrazinic residues in contaminated food sources, which at present include catfish [4], poultry muscle tissues and eggs, and pig liver, kidney, and rump muscle [5].

A related class of compounds, the hydrazides, are used in medicine; these include the anti-tuberculosis drug isoniazid [6], the anti-hypertensive and peripheral vasodilator drug hydralazine [7]. Isoniazid[1] (isonicotinic acid hydrazide) has been shown to produce lung tumors in mice [8] and to induce chromosome aberrations and sister chromatid exchanges in cultured rodent cells [9]. Hydrazides are also components in many complex drugs, such as nitrofurans [10], and they show up as breakdown products in the livers of animals treated with those drugs. They are used as antibacterial drugs in veterinary medicine. One of these, known as furazolidone [*N*-(5-nitro-2-furfurylidine)-3-amino-2-oxazolidone], contains two chemical rings: a nitrofuran-ring, responsible for the antibacterial and antiprotozoal activity, and an oxazolidone-ring (AOZ) which is a hydrazide.

As to the mode of toxicity of hydrazine, it is well known that methyl- or diethyl-hydrazine are quite sensitive to oxidation and data collected from in vitro studies suggest that they may serve as the electron donor for the reduction of oxygen [11], but there have been no equivalent studies for hydrazide derivatives. In view of the wealth of evidence pointing to the toxicity of hydrazides to animal cells, it is of primary importance for human health to determine the molecular mechanisms and biological implications of cellular reactions involving hydrazide compounds.

Data reported here showed that hydrazides as hydrazines produce oxygen radicals at various degrees of efficiency, which cause irreversible damage to the prosthetic group of proteins as well as degrade their polypeptide chain into small fragments as revealed by investigating cytochrome *c* and light harvesting complex (Lhcb) degradation upon hydrazide addition. The presence of atmospheric oxygen is necessary for the formation of reactive species.

2. Materials and methods

2.1. Reagents and materials

Reagent-grade phosphoric acid, trifluoroacetic acid (TFA), methanol, ethanol, as well as HPLC-grade water and acetonitrile, were obtained from Carlo Erba (Milan, Italy). Acrylamide, *N*,*N*′-methylene-bis-acrylamide, and all other reagents for SDS–PAGE were

[1] *Abbreviations used:* AOZ, 3-amino-2-oxazolidone; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-R·, carbon central adduct of 5,5-dimethyl-1-pyrroline *N*-oxide; ESI, electrospray ionization; ESR, electron spin resonance; HPLC, high performance liquid chromatography; Isoniazid, isonicotinic acid hydrazide; Iproniazid, isonicotinic acid 2-isopropyl hydrazide; Lhcb, light harvesting complex of photosystem II; MES, 2[*N*-morpholino]-ethanesulfonic acid; MS, mass spectrometry; PAGE, polyacrylamide gel elctrophoresis; PSII, photosystem II; RIC, reconstructed ionic current; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

purchased from Bio-Rad (Segrate, Italy). Sucrose, tricine, tris-hydroxymethylaminomethane (TRIS), and DMPO were purchased from Fluka (Milan, Italy). Hydrazine, isoniazid, and iproniazid were purchased from Sigma. For all experiments water was treated with chelax.

2.2. Measurement of oxygen consumption and ESR measurements

The oxygen consumption during the auto-oxidation of hydrazine and hydrazides was measured in a thermostat controlled (37 °C) water-jacketed glass vessel, fitted with a Clark electrode (Gilson, Paris, France) as reported previously [12]. The chemical under investigation was added to 1.8 mL of 50 mM sodium phosphate buffer at physiological value of pH 7.4, to give a final concentration of 10^{-5} M. The initial oxygen concentration was 230 μM.

The effect of various metal ions, Mn (II), Fe (II), Cu (II), Ni (II) or Co (II), on the auto-oxidation of hydrazides was determined by adding them, at a concentration of 20 μM to a solution of Iproniazid 10^{-5} M. For ESR experiments a Bruker ESP300 spectrometer equipped with a TE110-mode resonator, using 10-mW power at 9.79 GHz was employed. Spectra were recorded using 1.0-G modulation and 100-G scanning in 21 s.

2.3. Polyacrylamide gel electrophoresis

Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 12 % acrylamide gel (15 cm × 1.5 mm) containing 7 M urea, using a BRL (Bethesda Research Laboratories, Gaithersburg, USA) Model V16 vertical gel-electrophoresis system. Gels were run at room temperature (20 °C) for 6 h at constant current of 20 mA using 25 mM Tris–glycine buffer, pH 8.8, containing 3.5 mM SDS. Gels were fixed and stained for 2 h in a 5:1:4 (v/v) methanol–glacial acetic acid–water mixture, containing 0.1% (w/v) Coomassie blue.

2.4. Electrospray mass spectrometry measurements

In direct infusion mode, a dilute solution of the analyte is pumped into the electrospray source-ion trap Esquire 3000 plus (Bruker Daltonik, Germany) at a flow rate of 4 μL/min. For MS analysis with pneumatically assisted electrospray ionization, a spray voltage of 4 kV was employed. Protein mass spectra were recorded by scanning the range 50–300 amu.

2.5. Isolation of the thylakoid membrane of chloroplasts and light harvesting complex

Separation of the thylakoid membrane and light harvesting complex (Lhcb) from spinach leaves was performed as reported by Huber et al. [13].

2.6. Damage to cytochrome c

The analytical Waters Spherisorb 5 μm ODS1 C-18 columns were pre-equilibrated with 5% (v/v) aqueous acetonitrile solution containing 0.05 % (v/v) TFA and samples were eluted from the column with a gradient of 5–100% acetonitrile in 60 min. The flow rate was

1.0 mL/min. The HPLC-ESI-MS experiments were carried out with a System Gold HPLC unit (Beckman) and an ion trap Esquire 3000 plus (Bruker Daltonik, Germany).

The 1 mL/min flow through the analytical column was split post-column, with 50 μL/min entering the mass spectrometer, and 950 μL/min going to the UV- and fluorescence detector. Details of instrumental setup are given in [14].

3. Results

The toxicity of various hydrazides was tested on several proteins and the possible molecular mechanism has been investigated. For this purpose the compounds used were representative of the chemical forms commonly used in human and veterinary medicine. The choice of these chemicals was based on the fact that besides representing different chemical structures of the hydrazide families, these chemicals are in common use, such as the drugs isoniazid [6] and iproniazid [15] and the heterocyclic metabolite of furazolidone, called: 3-amino-2-oxazolidone (AOZ) [16]. Fig. 1 shows the structures of the chemicals tested. We did, in fact, test a much wider range of chemicals but since similar results were obtained for all we chose a representative sample to illustrate the case.

3.1. Auto-oxidation of hydrazides and hydrazine

To verify that atmospheric oxygen can react with both hydrazines and hydrazides, we measured the oxygen consumption of both chemicals exposed to air in the presence or absence of certain metals. Fig. 2A reports the oxygen consumption recorded by a Clark electrode of different hydrazides and hydrazine (10^{-5} M) exposed to air in the absence of metals. It can be observed that hydrazine consumes the most followed in decreasing order by iproniazid and isoniazid, while AOZ showed a very slow rate of consumption, where the presence of cyclic structures might be responsible for the observed retardation of the oxygen consumption process. It is worth remarking that with all chemicals tested oxygen consumption is strongly effected by the pH of the solution: alkaline pH increases the time course of oxygen uptake (inset of Fig. 2A), indicating that protons are released during the reaction of the chemicals with oxygen. Interestingly, the oxygen consumption decreases drastically if bottles containing pure chemicals were taken open at air for days, corrob-

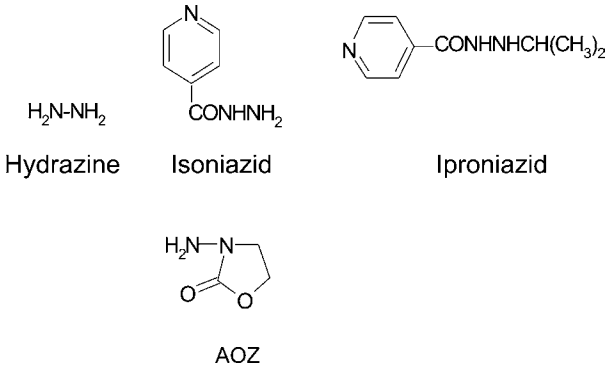

Fig. 1. Chemical structure of chemicals tested: hydrazine, iproniazid, isoniazid, 3 amino-2-oxalidone (AOZ).

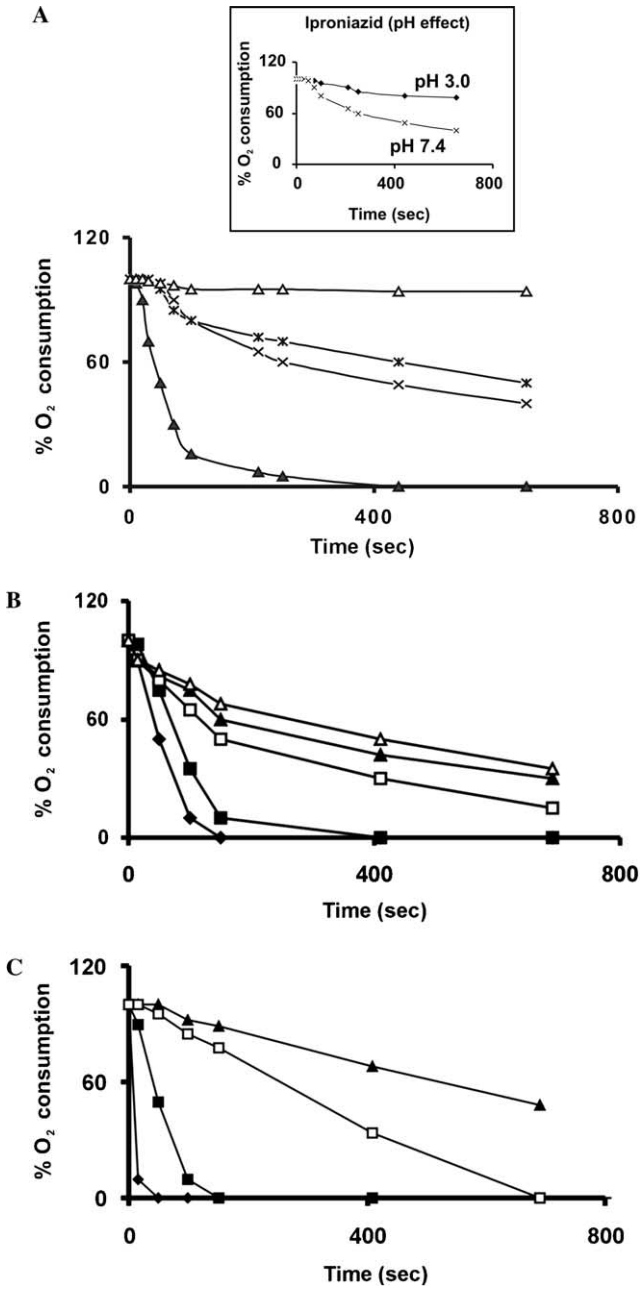

Fig. 2. O_2 consumption during auto-oxidation of hydrazine and different hydrazides in the absence or in the presence of metals. (A) Auto-oxidation of 10^{-5} M different hydrazides and hydrazine: hydrazine (▲), iproniazid (×), isoniazid (∗), AOZ (△). Inset shows the pH effect on the O_2 consumption of iproniazid. (B) Auto-oxidation of iproniazid 10^{-5} M in the presence of 20 μM of: $MnCl_2$ (♦), $CuCl_2$ (■), $CoCl_2$ (□), $NiCl_2$ (▲) or $FeCl_2$ (△). (C) Auto-oxidation of hydrazine (♦), iproniazid (■), isoniazid (□), AOZ (▲) in the presence of $MnCl_2$ 20 μM.

orating the hypothesis of a direct reaction between pure chemicals and atmospheric oxygen.

Addition of some metallic cations significantly accelerated the time course of oxygen consumption. Fig. 2B shows the effect of 20 μM of metal ions on O_2 consumption during the auto-oxidation of iproniazid (10^{-5} M). The order of catalytic ability in iproniazid auto-oxidation was as follows: Mn > Cu > Co > Ni > Fe. Fig. 2C shows the effect of Mn^{2+} 20 μM on O_2 consumption of different hydrazides. The time course of oxygen consumption for isoniazid and iproniazid is so rapid that it is unlikely to be the slow spontaneous hydrolytic release of hydrazine and isopropylhydrazine that is responsible for the reaction with the oxygen. In agreement, Fig. 3 shows that the molecular mass of iproniazid, measured by ion trap mass spectrometer either just after solubilization or after 3 h in solution, remains constant in the absence of oxygen (Figs. 3A and B). It was found that when there was no atmospheric oxygen present in the solution there was no change in molecular mass of chemicals. There were no lower molecular mass molecules detected, indicating that the structure of the hydrazide remains intact after solubilization. This led to the conclusion that oxygen consumption occurs as a result of hydrazide reacting directly with oxygen. Consistent with this hypothesis, it was observed that addition of oxygen gives rise to increasing peaks in the ESI spectra as a function of time (Fig. 3C). This is likely a consequence of oxygen radicals reacting with the entire molecule, producing dif-

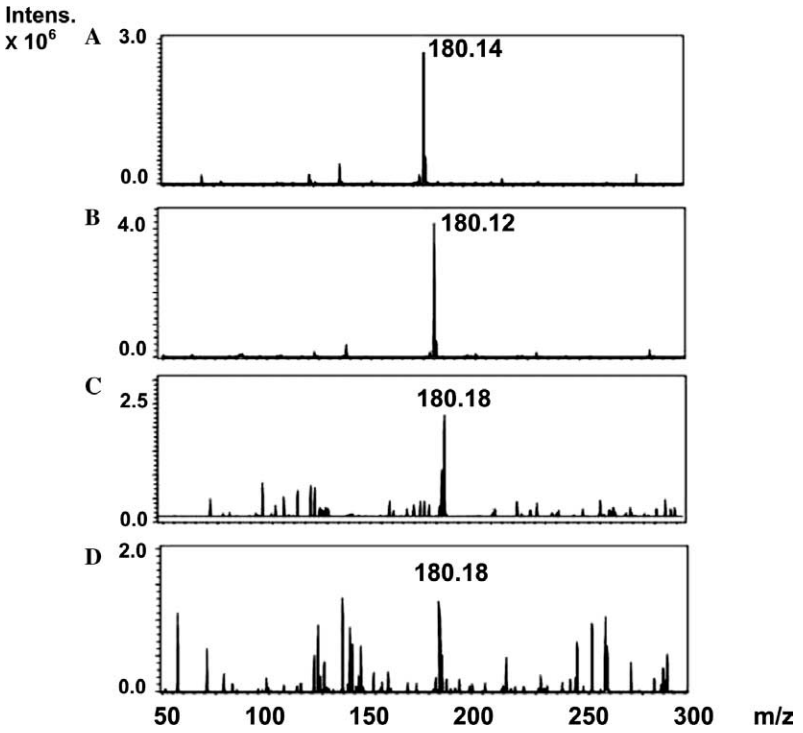

Fig. 3. Molecular mass of iproniazid measured just after its solubilization (A), after 3 h in solution without oxygen (B) and after 3 h under air in the absence (C) or in the presence of Mn^{2+} (D). The molecular mass measurements were performed by ion trap mass spectrometer equipped with an electrospray source.

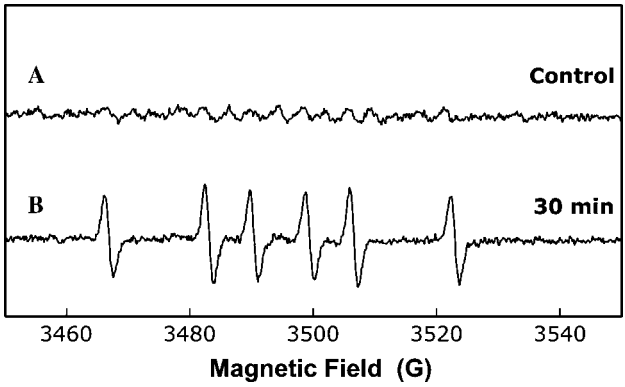

Fig. 4. ESR spectra of the radical adducts of DMPO produced during the auto-oxidation of the iproniazid at room temperature. The sample contained 10^{-5} M iproniazid, 1 mM DETAPAC, and 80 mM DMPO in 20 mM sodium phosphate buffer (pH 7.4). Control (A): spectrum recorded in the presence of iproniazid after 30 min of incubation (B).

ferent chemical compounds while the peak intensity corresponding to intact molecules decreases over time. In agreement to what observed above by Clark electrode, the presence of Mn^{2+} significantly increases degradation of the intact molecule, as shown in the ESI spectra of Fig. 3D.

Finally, electron spin resonance (ESR) was used to unequivocally confirm that radical species are produced when hydrazide derivatives react with atmospheric oxygen either in the presence or absence of heavy metals as was the case when iproniazid was incubated at room temperature in the presence of 80 mM DMPO, a known spin trap of free radical species. In the absence of oxygen no radicals were observed in any case, corroborating the hypothesis that oxygen plays a crucial role in radical species production. In the presence of oxygen, the result was that after 30 min incubation iproniazid was able to produce an ESR spectrum of a DMPO spin adduct (Fig. 4B). This adduct is formed more quickly in the presence of metals. Interestingly this signal increases with increasing oxygen concentration (data not shown). The spin adduct ($a^N = 16.1$ G, $a^H = 24.4$ G) is assigned to DMPO-$R^{\cdot}$ by referring to the reported constant [17], indicating that a carbon-centred radical is formed, probably on the hydrazide molecule. Similar evidences were observed with most of the hydrazides tested, corroborating the hypothesis that both hydrazide and hydrazine can serve as the electron donor for the reduction of oxygen. Although it is not easy to separate the two processes, further experiments are in progress in our laboratory to better clarify the molecular mechanism by which oxygen active species are produced.

3.2. Protein degradation induced by hydrazides

Addition of iproniazid to cytochrome *c* gives rise to optical variation of the cytochrome *c* heme group (left inset of Fig. 5), indicating that iron present in the heme group is rapidly oxidized. The same result was obtained with haemoglobin (Hb) forming metahaemoglobin (data not shown). Moreover, when cytochrome *c* solution is incubated with iproniazid and injected onto a reversed phase column it was possible to observe that the peak at 214 nm corresponding to cytochrome *c*, decreases in function of incubation times, thus suggesting

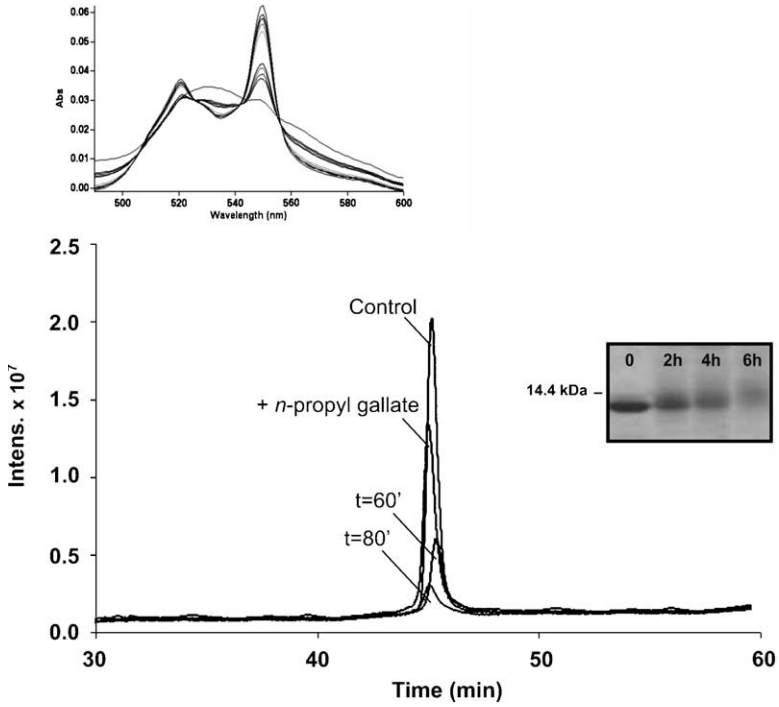

Fig. 5. Reconstructed ion chromatogram of cytochrome *c* before and after incubation with iproniazid for 60 and 80 min; incubation for 60 min was also performed with addition of *n*-propyl gallate 1 mM. Left inset reports the cytochrome *c* spectra recorded at different interval times after addition of iproniazid. Right inset reports the SDS–PAGE of the same sample incubated for longer times (hours). Iproniazid (10^{-6} M) was added to cytochrome *c* (10^{-5} M) at 37 °C in the presence of Mn^{2+} 20 μM.

that the protein is degraded (data not shown). However, to demonstrate unequivocally that the changes observed in the HPLC chromatogram are really due to fragmentation of the protein and not to optical absorption changes of chromophore, we coupled the HPLC on line with a mass spectrometer. Fig. 5 compares the reconstructed ion chromatogram, based on the individual ESI-MS spectra of the cytochrome *c* recorded initially, before any addition of compound, after incubation with iproniazid for 60 and 80 min, and upon addition of a radical scavenger. A significant decrease of the ionic current intensity was observed, corresponding to the cytochrome *c* peaks, which agrees with the optical decrease observed at 214 nm. However, it is worth remarking that the native protein is not degraded if incubation with hydrazides is performed in absence of oxygen or in the presence of *n*-propyl gallate, a well known scavenger of alkyl/alkoxyl radicals. Thus, it may be inferred that the solubilization of hydrazide into water in the presence of atmospheric oxygen, produces oxygen active species which, besides oxidizing the prosthetic group in a short period of time, also destroys the cytochrome *c* polypeptide chain over time. In agreement, SDS–PAGE of cytochrome *c* incubated at longer times (2–6 h) with iproniazid 1 μM reveals a concomitant and significant decrease of Coomassie staining (right inset of Fig. 5A) whit respect to the control which remained unchanged. However, by HPLC or by RIC, the protein degradation can be observed at just short incubation times, allowing for the investigation of the initial stages of the event.

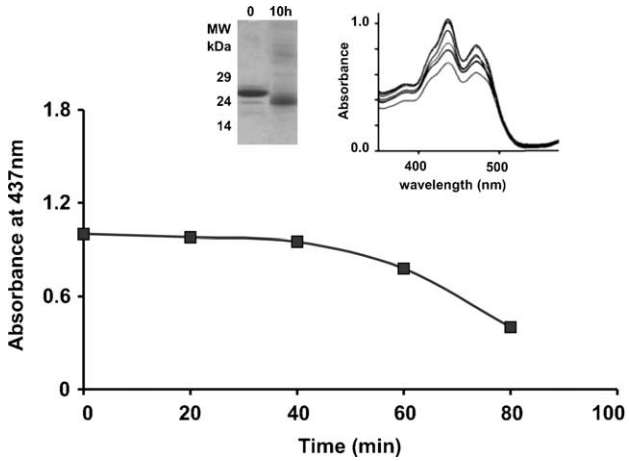

Fig. 6. Time course recorded at 437 nm of the photosystem II light harvesting complex inhibition, upon addition of 10^{-6} M iproniazid. Right inset shows the chlorophyll spectra of a mixture of light harvesting antenna recorded at different interval times after addition of 10^{-6} M iproniazid. Left inset reports the SDS–PAGE of the antenna proteins after 10 h of incubation with iproniazid.

To demonstrate that what observed in haemoglobin and cytochrome *c*, is not related to iron protein, but it is a general phenomenon, we extend the investigation to light harvesting proteins present in the photosystem II (Lhcb) of higher plants. These proteins contain chlorophyll as chromophore and Mg^{2+} as chelated metal [18]. The right inset of Fig. 6 shows the changes in chlorophyll absorption recorded upon addition of iproniazid 1 μM to a mixture of these antenna proteins, whereas Fig. 6 reports the time course recorded at 437 nm, which is much slower than that observed in the case of cytochrome *c*. SDS–PAGE of Lhcb subjected to longer incubation times (ten hours) shows a decrease in Coomassie staining (left inset of Fig. 6), as observed with cytochrome *c*. Both oxidation and degradation of light harvesting proteins is not observed in the absence of oxygen.

4. Discussion

The data presented here support the conclusion that hydrazides cause damage to proteins via a similar mechanism as hydrazine, although reaction rates vary. All these compounds share an N–N moiety, which is assumed to play an essential role in the irreversible damage to the prosthetic group of most enzymes and in causing degradation of the polypeptide chain of proteins. The key to the molecular mechanisms by which these compounds cause their irreversible effects may lie with the oxygen consumption recorded and ESR measurements, which revealed that hydrazides produce oxygen radicals at various degrees of efficiency. The different time course of oxygen consumption, which took the following order of decreasing rate: hydrazine > iproniazid > isoniazid, also reflected the different toxicity of the chemicals tested in the cytochrome *c* oxidation and chlorophyll oxidation reported here.

The mechanism which leads to the formation of active oxygen species during the auto-oxidation of both hydrazines and hydrazide, is likely to involve the following reaction:

$$\text{R-NHNH}_2 + \text{O}_2 \rightarrow \text{R-NH–}^{\bullet}\text{NH} + \text{O}_2^- + \text{H}^+$$

Our finding that oxygen consumption is strongly pH dependent is in agreement with this. Heterocyclic compounds containing an N–N moiety ''buried'' within the chemical molecule, such as AOZ, cause lesser damage than hydrazine, iproniazid and isoniazid that containing an N–N moiety in the substituent chain cause rapid damage to proteins. Clearly the presence of an alkyl or aryl substituent influences the reactivity. The reaction of hydrazides with oxygen is accelerated by the presence of metal ions, indicating that hydrazide can produce oxygen radicals both in the presence and absence of ion metals. This is in contrast to previous reports that the presence of a metal is a necessary condition for hydrazine to react with oxygen [19]. In agreement we observed that pure hydrazide chemicals taken open at the air, lose their reactivity towards oxygen was determined by the oxygen consumption measurements. However, the following order of catalytic effect on hydrazide-dependent protein damage, Mn (II) > Cu (II) > Co (II) > Ni (II) > Fe (II), is related to that of the accelerating effect on the O_2 consumption rate of hydrazide auto-oxidation. The catalytic effect of metal ions on hydrazide activity may be tentatively explained in terms of the formation of a complex between the metal ion and the hydrazide which favors the release of H^+ from the reactive nitrogen as follows:

$$(\text{Me}^{2+})\ \text{R-}^{\bullet}\text{NHNH}$$

This is suggested by the results obtained by Lim and Fagg [20] who reported the formation of Mn^{2+} ($\text{R-}^{\bullet}\text{NHNH}_2$) in Mn (II)-catalyzed auto-oxidation of aqueous hydrazine. They postulated that positive metal ions catalyse the reaction of the N–N moiety with oxygen, although by different mechanisms. However, irrespective of which type of active species is formed, all attack the prosthetic group as well as any polypeptide chain of proteins, resulting in complete degradation of it. This was clearly observed through the decrease of optical absorption at 214 nm, the decrease of reconstructed ions current (RIC) and the reduction in Coomassie staining in the SDS–PAGE electrophoresis of both cytochrome *c* and light harvesting proteins treated with hydrazide derivatives. Over time proteins are completely degraded by oxygen radicals, as previously remarked with the light harvesting proteins, which upon excitation of chlorophyll, produce singlet oxygen [21].

Interestingly, upon short time, RIC reveals a significant decrease of the ionic current intensity corresponding to the cytochrome *c* peaks, which is not confirmed by SDS, but agrees with the optical decrease observed. This would imply that the external part of the protein is the first to be attacked by active oxygen where ionizable amino groups are essentially located on and the aromatic amino acids contribute most to the optical absorption reading of a protein. Thus removal of the external hydrophilic portions of the proteins, rich in these, results in a marked fall in optical absorption and current ions, whereas SDS–PAGE is not sufficiently sensitive to reveal small changes in proteins. Moreover, monitoring of protein degradation by HPLC and MS, besides being more effective than SDS–PAGE electrophoresis, also allows quantitative estimation of the relative amount of each protein component. This is calculated from the area underlying each HPLC peak.

Taking the protein fragmentation into due consideration, the disappearance of protein in haemoglobin experiments reported by Runge-Morris et al. [22] was probably due to proteolysis, since our data has revealed that it is degraded chemically by the oxygen radicals produced during the oxidation of the heme group. On this note, it is reasonable to

assume that the increase in the free amino acid pool, recently observed in the urine of rats subjected to metabonomic investigations into hydrazine and hydrazide toxicity [23], is the result of aspecific protein degradation caused by both compounds.

In conclusion, hydrazides tested in this paper have been shown to damage proteins via a similar molecular mechanism as the hydrazines: the production of oxygen active species by reacting with atmospheric oxygen. Although this reaction does not require metal ions, the presence of such will accelerate it. It is significant that our experiments have yielded dramatic results in a matter of hours. When these same toxic residues remain intact, bound to animal or human tissues for months, possibly years, as in the case of treatment by 5-nitrofuran derivatives [24], it is clear that even slow reactions will have a measurable cumulative effect over such a time course. The cell contains numerous metal containing molecules that could enhance such reactions, not to mention the presence of metals accumulated in tissues through environmental pollution. Thus it is of interest to investigate in the near future the cellular damages produced by drugs containing an N–N moiety.

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