Oxidation and Nitrosation in the Nitrogen Monoxide/Superoxide System

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Based on the previous report of McCord and co-workers (Crow, J. P., Beckman, J. S., and McCord, J. M. (1995) Biochemistry 34, 3544–3552), the zinc dithiolate active site of alcohol dehydrogenase (ADH) has been studied as a target for cellular oxidants. In the nitrogen monoxide (NO)/superoxide (O2•-) system, an equimolar generation of both radicals under peroxynitrite (PN) formation led to rapid inactivation of ADH activity, whereas hydrogen peroxide and NO alone reacted too slowly to be of physiological significance. 3-Morpholino sydnonimine activated the enzyme with an IC50 value of 250 nM; the corresponding values for PN, hydrogen peroxide, and NO were 500 nM, 50 μM, and 200 μM. When superoxide was generated at low fluxes by xanthine oxidase, it was quite effective in ADH inactivation (IC50 (XO) ~ 1 milliunit/ml). All inactivations were accompanied by zinc release and disulfide formation, although no strict correlation was observed. From the two zinc thiolate centers, only the zinc Cys4His center released the metal by oxidants. The zinc Cys6 center was also oxidized, but no second zinc atom could be found with 4-(2-pyridylazo)resorcinol (PAR) as a chelating agent except under denaturing conditions. Surprisingly, the oxidative actions of PN were abolished by a 2–3-fold excess of NO under generation of a nitrosating species, probably di-nitrogen trioxide. We conclude that in cellular systems, low fluxes of NO and O2•- generate peroxynitrite at levels effective for zinc thiolate oxidations, facilitated by the nucelophilic nature of the complexed thiolate group. With an excess of NO, the PN actions are blocked, which may explain the antioxidant properties of NO and the mechanism of cellular S-nitrosations.

There is increasing evidence that redox reactions at proteins can modify enzyme activities and thus represent a new pathway in the cellular signaling network (reviewed in Refs. 1–5). Molecular oxygen must eventually be involved in such modifications, which can consist of thiol oxidations leading to sulfinic acids or disulfides, sulfoxidation of methionine, nitration of tyrosines, or carbonyl formation (6, 7). Bolus additions of H2O2, organic hydroperoxides, or peroxynitrite (PN)1 have frequently been used to study such oxidations, but in most cases, the effective concentrations are nonphysiological and can only partly reflect the physiological steady-state levels of oxidants. We have recently obtained evidence that PN can control vascular tone already at submicromolar concentrations by nitration of a tyrosine residue in prostacyclin synthase under physiological conditions (8). Simultaneous low-level generation of nitrogen monoxide (NO) and superoxide radicals (O2•-) in endothelial cells proved to be sufficient for this oxidative modification (9, 10), although it has been argued that physiological levels of both radicals are not sufficient for tyrosine nitration (11, 12). Meanwhile, literature data show that PN generated from superoxide and nitrogen monoxide can nitrate tyrosine (13–15). Moreover, this discrepancy found an explanation in a series of studies proving that the sensitivity and selectivity of heme proteins for tyrosine nitration reside in an autocatalytic role of the metal ion (16, 17). By forming transition metal intermediates from cellular levels of PN, neighboring tyrosines can be selectively attacked without affecting other tyrosines randomly.

Following this concept of PN action under physiological conditions, we now extend oxidations by PN also to cysteines. Free and also protein-bound thiols are preferred targets of PN, especially when they are present in their thiolate forms (18–22). Then a direct two-electron oxidation of the sulfur by the electrophilic PN molecule can take place. It has been reported that the essential ZnCys4His center of alcohol dehydrogenase (ADH) can be oxidized by micromolar bolus concentrations of PN (23) and also by NO (24) and hypochlorite (25). This can be interpreted as a catalytic action of Zn2+ by converting thiol groups after deprotonation to the more reactive thiolate form. Such zinc thiolate centers may be the subject of a redox regulation by being reversibly oxidized and reduced as part of a signaling mechanism in oxidative stress (26–28). Therefore, ADH provides a model in which the oxidation of the active site Zn2+ dithiolate center can be studied in the NO/O2•- system by monitoring the enzyme activity. In the present work, we confirm the validity of this model and report on chemically surprising and biologically relevant changes in the oxidizing, nitrating, and nitrosating potential of the system when varying the relative flux rates of NO and O2•-.

EXPERIMENTAL PROCEDURES

Materials—PN was synthesized from NO and O2•- according to the method of Kissner et al. (29). SIN-1 and spermine NONOate were purchased from Tocris Cookson, United Kingdom. diethylamine NONOate, ethanamine, N-ethyl compound with 1,1-diethyl-2-hydroxy-2-nitrosohydroxine (1:1); SOD, superoxide dismutase; XO, xanthine oxidase; PAR, 4-(2-pyridylazo)resorcinol; DTBP, 2,2′-dithio-bipyridine; HPLC, high pressure liquid chromatography.

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‡ The abbreviations used are: PN, peroxynitrite; ADH, alcohol dehydrogenase; SIN-1, 3-morpholino sydnonimine; spermine NONOate, (Z)-1-[N-(3-amino propyl)-N-[4-(3-amino propylammonio)butyl]-amino]-dlszen-1-ium,1,2-dioate; diethylamine NONOate, ethanamine, N-ethyl compound with 1,1-diethyl-2-hydroxy-2-nitrosohydroxine (1:1); SOD, superoxide dismutase; XO, xanthine oxidase; PAR, 4-(2-pyridylazo)resorcinol; DTBP, 2,2′-dithio-bipyridine; HPLC, high pressure liquid chromatography.
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purchased from Calbiochem, and diethylamine NONOate was purchased from Cayman Chemical Co. (Ann Arbor, MI). Stock solutions of PN (185 mM in 0.1 M NaOH), SIN-1 (100 mM in 0.1 M HCl), and diethylamine NONOate and spermine NONOate (100 mM in 0.1 M NaOH) were kept at -70 °C for months without serious losses. XO (grade 8, from Sigma) and Cu,Zn-SOD were obtained from Roche Molecular Biochemicals. All other reagents were of analytical grade.

ADH Activity Test—The activity of ADH was measured on an Aminco DW-2a UVVis spectrophotometer in the dual wavelength mode. A solution of ADH (3.3 nM) in 0.1 M potassium phosphate buffer, pH 7.4, was placed in a cuvette equipped with a magnetic stirrer, and then NAD+ (300 μM) was added, and the temperature of the solution was adjusted to 37 °C. The reduction of NAD+ to NADH was started by addition of ethanol (172 μM) from a syringe through a septum to the stirred solution. The formation of NADH was followed at 340 versus 400 nm, and the specific activity of ADH was calculated for the first 30 s of the measurements in the linear region of the curves by using an extinction coefficient for NADH of ε340 = 6220 m⁻¹ cm⁻¹ (23). The specific activity of freshly prepared ADH was 65 ± 3 μmol min⁻¹ mg⁻¹ (units/mg). For all experiments, the same stock solutions of ADH (3.3 mM in water) and NAD+ (3 mM in buffer) were used, aliquots of which were frozen at -70 °C.

Induction of ADH with Peroxynitrite, Hydrogen Peroxide, or Potassium Superoxide—To test the effects of oxidants on the activity of ADH, the enzyme (33 mM) without NAD+ was preincubated with these oxidants at pH 7.4 and 37 °C. PN (0.1–10 μM) was added by a Vortex mixer to a solution of ADH and incubated for 5 min at pH 7.4 and 37 °C, and then all samples were kept on ice until they were measured. H2O2 (25–1000 μM) was added with ADH for 1 or 2 h at pH 7.4 and 37 °C, and aliquots were taken after defined time intervals. The effects of catalase (5–500 units/ml) and Cu,Zn-SOD (100 and 500 units/ml) on this inactivation by H2O2 (0.5 or 1 mM) were measured in the presence of these proteins during incubation. Potassium superoxide (K2O2) (2.5–100 μM from a freshly prepared stock solution in Me2SO) was incubated for 1 min before the activity test. The final amount of Me2SO was kept below 2% to prevent activity loss by this compound.

Incubation of ADH with SIN-1, Xanthine Oxidase, Spermine NONOate, or Diethylamine NONOate—SIN-1 (0.1–5 μM), which under aerobic conditions generates PN from NO and O2 with a half-life of about 1 h at pH 7.4 and 37 °C (11, 30), was incubated for 2 h with ADH. The effects of catalase (100 and 500 units/ml), Cu,Zn-SOD (1–500 units/ml), and NO (1–25 μM) on the inactivation of ADH by SIN-1 (5 μM) were tested by addition before incubation. Spermine NONOate (5–100 μM) of diethylamine NONOate (50–1000 μM), which both thermally decompose and yield NO (with half-lives of 40 min and 2 min, respectively, at pH 7.4 and 37 °C (31)), were incubated for 2.5 and 1 h, respectively, with ADH. These incubations were done in the presence and absence of oxygen. XO (0.625–20 million units/ml) as an enzymatic source for O2 (32, 33) was incubated with ADH for 0.5 and 1 h at pH 7.4 and 37 °C in a buffer that contained 20 mM potassium phosphate buffer at pH 6.8. After this treatment, the samples were then buffer-exchanged into 50 mM NaCl, pH 7.0, on Sephadex G-25 fast desalting column (Amersham Biosciences, Inc.) and concentrated to 50 μl in Microcon-10 centrifuge tubes (Amicon). 10-μl aliquots were spotted onto glass coverslips, dried under vacuum, and stored in argon atmosphere. Raman spectra were taken on a Jobin Yvon LabRAM spectrometer (courtesy of Jobin Yvon; GmbH, Bensheim, Germany) with 633 nm excitation from a helium-neon laser.

Quantitation of 4-Nitrosophenol by UV/Vis Spectroscopy and HPLC—The formation of 4-nitrosophenol (pK = 6) in reactions of phenol (5 mM) with SIN-1 (200 μM) and/or spermine NONOate (100 μM) was monitored spectrophotometrically at 400 versus 500 nm over a time period of 2 h in 0.1 M potassium phosphate buffer at pH 7.4 and 37 °C (37). Its yield in these samples was determined by HPLC as described previously (38).

RESULTS

Inhibition of ADH Activity by Nitrogen Monoxide, Bolus Additions, and Detection of Thiol Oxidation—In ADH, the ZnCys₃His cluster is essential for enzyme activity (39), allowing correlation of the loss of activity with the oxidation by PN (Fig. 1). By bolus additions of PN, ADH became inactivated with IC₅₀ values of 0.5 μM PN and thus with similar sensitivity as reported previously (23) because such values are dependent on the target concentrations. This inactivation was complete at about 4 μM within 1 min, in agreement with the short half-life of PN of about 1 μM (23).

Because under cellular conditions PN originates from the fast combination of NO and O₂⁺ (29, 41), we used SIN-1 as a suitable agent for the simultaneous generation of both radicals in equimolar concentrations (11, 30). According to the clear-cut results in Fig. 2, the inhibition occurred with an IC₅₀ value of about 0.25 μM and hence perfectly matched the data from bolus additions of PN. The presence of Cu,Zn-SOD abolished the inhibition (Fig. 2, inset), and NO alone generated by spermine...
NONOate required about 200 μM for half-inhibition (Fig. 3). This weak oxidizing power of NO was not significantly influenced by the presence of dioxygen. At concentrations of 1–5 μM spermine NONOate, NO even caused a slight increase in ADH activity, but only in deoxygenated solutions. From this one can conclude that NO is not an effective oxidant for the zinc di-thiolate center of ADH and that the simultaneous generation of NO and O2 from SIN-1 is required for an efficient inactivation of the protein.

**Inhibition of ADH Activity by Peroxynitrite**—In a similar way controls were run to show whether O2− alone would be sufficient to cause inactivation. Bolus additions of KO2 and incubation times of only 1 min (to avoid the slow inactivation by H2O2) resulted in no inhibition (data not shown). However, because the release of O2− by SIN-1 occurs by a slow aerobic decay reaction over more than 1 h, the continuous generation of O2− by the XO/hypoxanthine system provided a better control (Fig. 4). Such conditions caused inactivation in a time-dependent (Fig. 4, inset) and XO concentration-dependent fashion (Fig. 4). The presence of Cu,Zn-SOD and catalase was used to differentiate between O2− and H2O2-dependent oxidations (Fig. 5). The varying results obtained from bolus additions of KO2 and from O2− generation by XO may be explained by the high velocity of disproportionation with bolus additions of O2− (42).

In the presence of Cu,Zn-SOD, almost 40% of the enzyme could be prevented from inactivation, and in the presence of catalase, close to 50% of the enzyme could be prevented from inactivation. The addition of both together resulted in almost 100% restoration of activity. This allows the conclusion that O2−, at least under constant generation, is an inactivating agent by itself. H2O2 as an oxidant requires quite high concentrations or long incubation times to exert its effects, and its concentration for half-maximal inhibition after 2 h was around 25–50 μM (Fig. 6). As expected, catalase already blocked the inactivation at low levels (Fig. 6, inset), whereas Cu,Zn-SOD did not (data not shown).

**Inhibition of ADH Activity by Simultaneous Generation of Superoxide and Nitrogen Monoxide**—An interesting observation was made when the O2−-generating system XO/hypoxanthine was supplemented with increasing concentrations of
spermine NONOate to vary the O$_2$/NO ratio (Fig. 7A). At a fixed rate of O$_2$ generation (which by itself caused 15% inactivation), upon the addition of spermine NONOate the activity of ADH first decreased steadily and reached a minimum at 4–6 μM spermine NONOate. According to calculations from our own measurements, this occurred at an about equal rate of O$_2$ and NO production. The initial formation rates in the XO system (0.5 milliunit/ml and 1 mM hypoxanthine) and from spermine NONOate (5 μM) were 0 ± 0.2 nmol/s for O$_2$ and 2.4 ± 0.1 nmol/s for NO at pH 7.4 and 37 °C. Cu,Zn-SOD at concentrations above 100 units/ml could block the inhibition almost completely at this minimum (Fig. 7B). The protective effect of Cu,Zn-SOD (2.5–250 units/ml) in the XO (0.5 milliunit/ml)/spermine NONOate (5 μM) system was investigated under the same conditions. Data represent the means ± S.D.

Oxidative Zinc Release from ADH and Thiol Oxidation—With regard to the mechanisms of oxidative ADH inactivation—

One mol of spermine NONOate will produce 2 mol of NO by thermal decomposition.
The protective effect of nitrogen monoxide released by spermine NONOate (1–25 μM) on the complete inactivation of ADH (33 nM) by SIN-1 (2 μM) was investigated at pH 7.4 and 37 °C (incubation times were 2 h). Data represent the means ± S.D.

**Table I**

Formation of 4-nitrosophenol by NO, SIN-1, and both together

| System                | 4-Nitrosophenol (μM) |
|-----------------------|----------------------|
| Spermine NONOate (100 μM) | 9.37 ± 1.46          |
| SIN-1 (200 μM)        | 9.38 ± 1.43          |
| Both                  | 46.55 ± 5.71         |

Previous work on PN action had shown a release of Zn^{2+} as a consequence of oxidation of the zinc dithiolate center in ADH (23). Because the release of zinc can easily be followed by complexation with the chelator PAR, we have monitored the formation of the Zn(PAR)_{2} complex spectrophotometrically between 450 and 550 nm. The time-dependent release of Zn^{2+} under the influence of SIN-1, H_{2}O_{2}, and/or spermine NONOate is shown in Fig. 9. It is evident that NO is ineffective in zinc release, H_{2}O_{2} is still a sluggish oxidant, superoxide is quite effective, and SIN-1, at a concentration of 250 and 1000 μM, is highly effective. The efficacy of the NO/O_{2} system was close to that of SIN-1. These data qualitatively follow the inactivation results and even match quantitatively for SIN-1, if one takes into account the 300-fold higher ADH concentrations in these samples (10 μM instead of 30 nM) and the fact that for PN the target concentration directly influences the IC_{50} values. The results obtained from additions of Cu,Zn-SOD and/or catalase allowed us to identify PN as the most effective zinc-releasing oxidant (data not shown).

To obtain a more direct comparison between zinc release and activity loss, one has to take into account the presence of a second zinc thiolate center (ZnCys_{6}), which is not involved in catalysis but may have structural significance (23, 39). Analysis of the zinc content by inductively coupled plasma mass spectrometry resulted in 2 zinc atoms/ADH subunit, and hydrolysis with hydrochloric acid and subsequent PAR complexation also gave the same value (1.96 zinc atoms/subunit). However, we could not observe the direct extraction of zinc from the ZnCys_{6} cluster by PAR as previously reported (23). This second zinc atom, which is not related to the enzyme activity, even required drastic conditions to be released. Based on these values of 2 zinc atoms/subunit, one observes the release of one mol of Zn^{2+} upon titration with SIN-1 (Fig. 10; see also Fig. 9). This maximal zinc release was already achieved by the addition of 300 μM SIN-1. Higher concentrations of SIN-1 did not result in increased zinc release but rather decreased it (data not shown). H_{2}O_{2} had to be used at a concentration of 10 mM to inactivate ADH (10 μM) completely, but it only caused the release of 0.5 zinc atom/subunit, whereas PN (10 μM) caused the release of 1.4 zinc atoms/ADH monomer (data not shown). Although the two parameters change almost inversely, the inactivation process seems to be somewhat faster than the zinc release. This may either be due to an additional inactivation mechanism (affecting about 20% of the zinc release) or because the zinc release does not exactly follow the oxidation of the two thiolate residues. The main mechanism, however, consists of the oxidation of the thiolate ligands because at the maximum zinc release (250 μM SIN-1), no thiol groups are left (data not shown).

Further evidence for this assumption provided the time-dependent inactivation of ADH by the thiol group determination compound DTBP, which was accompanied by zinc release (data not shown). Half oxidation of the thiol groups of ADH (10 μM) was observed at around 25 μM SIN-1, 5 mM H_{2}O_{2}, or 7.5 milliunits/ml XO. 500 μM NONOate caused only 27% of thiol oxidation, thus confirming that the ZnCys_{6}His center resists nitrosation and oxidation by NO.

**Fig. 9. Oxidative zinc release from ADH after treatment with varying oxidants.** The zinc release from ADH (10 μM) by time-dependent oxidation with different oxidants was measured by PAR (100 μM) at pH 7.4 and 37 °C. ADH was incubated with the oxidants, and the zinc release was measured by addition of PAR in aliquots, which were taken after the indicated time periods. □, 1 mM SIN-1; ●, 250 μM SIN-1; ★, XO (25 milliunits/ml)/spermine NONOate (100 μM); ▼, 10 mM H_{2}O_{2}; ♦, XO (25 milliunits/ml); ◆, spermine NONOate (250 μM).

**Fig. 8. Prevention of SIN-1-dependent inactivation of ADH by excess nitrogen monoxide formation from spermine NONOate.** Phenol (5 mM) was incubated for 2 h with SIN-1 and/or spermine NONOate in 0.1 M potassium phosphate buffer, pH 7.4, at 37 °C. The reactions were performed directly in a cuvette, and the kinetics were measured at 400 nm versus 500 nm. After complete decay of the donors, the yields of 4-nitrosophenol were determined by HPLC. Mean values were determined from three independent measurements.
and a similar way as in under cellular conditions (12), can oxidize zinc thiolate centers, effective (11). This raised doubts regarding whether tyrosine SIN-1 (5 thiol groups by PN can be greatly enhanced by Zn\(^{2+}\). This reaction can inactivate yeast ADH by oxidation of the ZnCys\(_2\)His center is required for activity and can serve as a target for PN. Inactivation of the enzyme occurred with an IC\(_{50}\) value of 0.25–0.5 \(\mu\)M PN, regardless of whether a bolus addition was used or a steady-state was generated. Such concentrations, however, are dependent on the concentration of the target or on the presence of other potential reaction partners; therefore, discussions on a possible physiological relevance have to await additional studies.

Our results have some additional implications. The oxidative release of 1 zinc atom/ADH monomer could be shown, although 2 zinc atoms are present in the ADH subunit according to inductively coupled plasma mass spectrometry analysis. The second Zn\(^{2+}\) could be partially released by a large excess of PN (1.4 zinc atoms/subunit) or completely released after protein hydrolysis (1.96 zinc atoms/subunit). According to the literature, in the native state, the second Zn\(^{2+}\) is bound to four other Cys residues (23), but these were not detectable after oxidative release of the first zinc from the active site. It remains a possibility that oxidation of this ZnCys\(_4\) center moved the zinc to another binding site with sufficiently high affinity to resist complexation by PAR. Further evidence for this came from the observation that SIN-1 at a concentration of 25 \(\mu\)M already oxidized 50% of the thiols but only released 0.3 zinc atom/ADH subunit. Qualitatively, the same results were found with \(\text{H}_2\text{O}_2\), which oxidized 50% of the thiols at a concentration of 5 mM, but only 0.2 zinc atom/ADH subunit was released under these conditions. Moreover, the direct formation of disulfide bonds could be monitored by Raman spectroscopy. For the oxidation of zinc finger motifs lacking a direct connection to activities, this might be a convenient method for the detection of oxidations at these potential targets for redox regulation.

Except for PN, other reactive oxygen and nitrogen species were examined but gave very different results. \(\text{H}_2\text{O}_2\) turned out to be a slow oxidant and certainly per se is not able to cause inactivation under cellular conditions. Also, NO could not inactivate ADH, although the formation of S-nitroso compounds by NO and even the reaction with zinc fingers have been reported (24). On the other hand, \(\text{O}_2^-\) which is not considered a strong oxidant and even has reducing properties, was quite sufficient for inactivation. In all cases, the loss of activity was paralleled by Zn\(^{2+}\) release and thiol oxidation, which allowed us to exclude mechanisms other than oxidation of the ZnCys\(_4\)His center. Thus, \(\text{O}_2^-\) can oxidize zinc thiolate centers, which is difficult to understand from its stoichiometry, unless Zn\(^{2+}\) as a Lewis acid can stabilize \(\text{O}_2^-\) in a similar way as in Cu,Zn-SOD for further reaction with a second \(\text{O}_2^-\) molecule. Other examples of superoxide-mediated enzyme activity modifications are known (47) and may be mechanistically related to the reaction observed here.

One of the most relevant findings was the blocking of PN action by an excess of NO. A 2–3-fold excess of NO over \(\text{O}_2^-\) abdolished the strong oxidations seen with PN or under equimolar generation of \(\text{O}_2^-\) and NO. It is known that PN can be scavenged by NO, but it came as a surprise that this reaction proceeds in an extremely rapid fashion. The PN anion clearly does not react with NO unless dioxygen is present (43). The formation of a nitrosating species, probably \(\text{N}_2\text{O}_3\) (43), could be confirmed by the nitrosation of phenol. Therefore, it may be suggested that this reaction is responsible for the antioxidative properties of NO. This sheds new light on the induction of NO-synthase II, which provides higher fluxes of NO in cellular systems. Consequently, not only thiol oxidations but also methionine sulfoxidations and tyrosine nitrosations would be down-regulated. Indeed, in a parallel study, we could block nitrosations at P450 proteins with an excess of NO over \(\text{O}_2^-\).

It should be pointed out that conflicting data in the literature on the use of SIN-1 as a PN-generating agent can be explained by this finding. If, in cellular systems, trapping of superoxide...
by NO-synthase, SODs or antioxidants shifts the NO/O$_2$ ratios to values greater than 2 or 3, then the actions of PN are eliminated. A limitation in the oxygen supply may also have the same effect. This is likely to happen if SIN-1 concentrations close to or greater than the dioxygen levels are used. SIN-1 should not be used at concentrations greater than 300 $\mu$M in air-saturated solutions because it could also switch to a NO donor under anaerobic conditions. An excess of NO could not only eliminate PN actions but even prevail nitrosating conditions. In vivo, nitroso derivatives could be the consequence, which, in view of the upcoming regulatory properties of S-nitroso derivatives, would be an attractive new property of the NO/O$_2$ regulatory system.

In summary, the oxidation of the Zn$^{2+}$ thiolate cluster in ADH not only serves as a suitable model for redox regulations involving zinc finger proteins but also unravels new chemical properties of the NO/O$_2$ system. One clearly has to take into consideration the participation of metal catalysis in PN action at physiological levels and the fine tuning of the relative rates of NO and O$_2$ fluxes that lead to sudden changes in the nature of reactive intermediates. As outlined recently, the different sources for O$_2$, their compartmentalization, and the presence of antioxidants and SODs will be as important as the different sources for NO and their regulatory factors. Thus, the NO/O system turns out to be a major, but not easily handled, player in the cellular signaling network.

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