EPR spectroscopy of putative enzyme intermediates in the NO reductase and the auto-nitrosylation reaction of Desulfovibrio vulgaris hybrid cluster protein

Wilfred R. Hagen

Department of Biotechnology, Delft University of Technology, Delft, the Netherlands

Correspondence
W. R. Hagen, Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629HZ Delft, the Netherlands
Tel: +31 15 2785051
E-mail: w.r.hagen@tudelft.nl

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The hybrid cluster protein (Hcp) contains a unique 4Fe cluster that is a hybrid of \( \mu \)-S and \( \mu \)-O bridges. Escherichia coli Hcp has recently been found to carry NO reductase activity as well as \( S \)-nitrosylation activity in NO-based signaling. In other species, the physiological activity has not been established. No reaction mechanism of any Hcp has been proposed. Here, we show that Desulfovibrio vulgaris (Hildenborough) Hcp has nitric oxide reductase activity with benzyl viologen as electron donor. With EPR spectroscopy, we identify three unexpected putative reaction intermediates: both in reduced and oxidized Hcp, dinitrosyl iron complexes are formed. Also, the hybrid cluster in reduced Hcp, but not in oxidized Hcp, binds the product N\(_2\)O. Possible implications for a reaction mechanism are discussed.

Keywords: Desulfovibrio; EPR; hybrid cluster protein; nitric oxide; nitrosylation; NO reductase

The hybrid cluster protein (Hcp) received its name from the fact that one of its prosthetic groups is a 4Fe cluster with both \( \mu \)-oxo and \( \mu \)-sulfido bridges between the irons [1] (Fig 1A,B). Hcp occurs widely in archaeal, bacterial, and eukaryal microorganisms [2,3]. In Escherichia coli, Hcp has been reported to exhibit two key activities: it is an NO reductase [4], and it has also been reported to be an auto-\( S \)-nitrosylase or \( S \)-nitrosothiol (SNO) synthase [5].

Hybrid cluster protein was accidentally isolated from Desulfovibrio vulgaris, substrain Hildenborough, three decades ago as an iron-sulfur protein with unidentified biological function but with an unusual EPR signal unprecedented in metalloprotein biochemistry [6]. In the first decade after its discovery, Hcp was extensively scrutinized with a range of spectroscopies including optics [7], EPR, Mössbauer, resonance Raman, magnetic circular dichroism, EXAFS ([1] and refs therein), ESEEM [8], paramagnetic NMR (unpublished), which resulted in an impressive butterfly collection of data but no clue as to the protein’s biological function. In the ensuing decade, Hcp was equally extensively scrutinized with X-ray crystallography [1,9–13] resulting in high-resolution data on its structure, including that of an unprecedented iron-oxo-sulfur cluster in several oxidation states but not in a specific proposal as to its putative function.

In the last one-and-a-half decade combined physiological and genetic studies have specifically aimed at pinning down Hcp’s function in the cell and these efforts have brought a continuous wave of aha-experiences and refutations. Initially, hydroxylamine reductase activity, the two-electron reduction of NH\(_2\)OH to NH\(_3\), was detected for E. coli Hcp but with unphysiologically high \( K_M \) values [14]. Subsequently, peroxidase activity, here the two-electron reduction of H\(_2\)O\(_2\) to two H\(_2\)O, was reported for both E. coli Hcp and Desulfovibrio desulfuricans, substrain ATCC 27774, Hcp, but specific activities were low [15]. More recently, following earlier indirect indications [16], NO

**Abbreviation**
BV, benzyl viologen; Hcp, hybrid cluster protein; HCuO, heme-copper oxidases; SNO, \( S \)-nitrosothiol.
reductase activity, the two-electron reduction of two NO molecules to one N₂O, was reported for E. coli Hcp with a very low apparent \( K_M \) for NO [4]. Very recently these studies have taken a rather different turn in a report on E. coli Hcp as the major auto-S-nitrosylating system, that is, an enzyme that accepts NO from a donor protein and uses it to nitrosylate one of its own cysteine residues, whereupon the SNO functionality may in turn be accepted by a transnitrosylase for propagation of signaling [5]. This study also concluded, based on single Cys mutations, that free Cys residues in E. coli Hcp are not involved in nitrosylation, thus implying one of the metal coordinating Cys, but it did not address the question how a cluster retains its integrity once one of the Fe-coordinating Cys are nitrosylated.

Thirty years down the line our present knowledge of Hcp can be summarized as follows: extensive data sets are available on spectroscopic properties, crystal structures, genetic context, and putative physiologically relevant activities; however, all this information has thus far not provided any insight into the working mechanism of the Hcp as enzyme. The present study is proposed to be a first step toward the resolution of this remaining challenge.

The following details are of relevance. Hcp proteins have been purified to homogeneity from five sources: the sulfate-reducing bacteria Desulfovibrio vulgaris [6] and D. desulfuricans [17], the enterobacterium E. coli [2], the photosynthetic purple bacterium Rhodobacter capsulatus [18], and the hyperthermophilic archaeon Pyrococcus furiosus [19]. Crystal structures have only been determined for the two Desulfovibrio Hcp’s [1,10–13]. The proteins contain a regular [4Fe-4S] cluster and a hybrid cluster. The E. coli Hcp differs from the Desulfovibrio ones in that the [4Fe-4S] cluster appears to be replaced by a [2Fe-2S] cluster as indicated by EPR spectroscopy [2]. Another, more consequential, difference is that the structural gene hcp in E. coli is found in a short operon of two genes in which the second gene, hcr, encodes an NADH-dependent reductase containing an FAD and a [2Fe-2S] cluster [2]. In other words, the natural redox partner for E. coli Hcp has been identified. The P. furiosus protein has a single-stretch 13 kDa deletion presumably contributing to its thermostability. The EPR signal putatively ascribed to its reduced hybrid cluster exhibits much reduced g- anisotropy compared to the other three Hcp’s [19]. The R. capsulatus Hcp has only been characterized in terms of its optical spectrum [18].

The hybrid cluster occurs in four stable oxidation states in D. vulgaris Hcp [20], in D. desulfuricans Hcp [21], and in E. coli Hcp [2]. These states are labeled ‘3+’ through ‘6+’ based on the early misconception that the hybrid cluster would be a [6Fe-6S] cluster [20]. Corrective re-numbering is at present not possible because the formal charges of the four states of the hybrid cluster are not accurately known. A functional re-naming might be: reduced, semi-reduced, oxidized, and super-oxidized, as a reflection of the idea that the cluster goes through its lowest three redox states in NO reduction catalysis (involving two electrons) and...
that the fully (or super-) oxidized state may not be physiologically relevant.

High-resolution x-ray crystallography has shown that the structure of the hybrid cluster in Desulfovibrio Hcp’s undergoes a very drastic structural change upon reduction from the 4+(semi-reduced) to the 3+(reduced) state as illustrated in Fig 1C, D.

The two bridging oxygens (O8 and O9) as well as the bridging ‘X’ (most likely also an oxygen) in the semi-reduced state have disappeared in the reduced state and have been replaced by the single ‘Y’ (modeled as oxygen but possibly a diatomic entity [12]). Also, the persulfido sulfur of Cys406 has undergone a large movement of 3.6 Å and is now a sulfido sulfur; similarly, Fe8 has undergone a large movement of 2.1 Å [12]. The structure of the hybrid cluster in the 5+(oxidized) state is essentially identical to that in the 4+ state except that the position of ‘X’ appears to be less well defined with two substoichiometric occupancies [10,11]. Note that the hybrid cluster in literature is usually referred to as [4Fe-2S-2O], and sometimes as [4Fe-2S-3O], but that a more accurate description for the 4+ and 5+ states would be [4Fe-2S-S*-3O] in which S* stands for persulfido sulfur; and the structure for the 3+ state would be [4Fe-3S-(‘Y’)] where the parentheses are to indicate that ‘Y’ is within hydrogen bonding distance of Fe7 and Fe8 but too far away for a bona fide coordination bond [12]. How these complex structures and their complex redox interchange are related to biological function is presently not known.

Incited by two recent publications documenting NO reductase activity [4] and S-nitrosylation activity [5] for E. coli Hcp, we have tried to make a first ingress into studies of the reaction mechanism(s) of Hcp by EPR, monitoring effects on the oxidized and reduced hybrid cluster of incubation with substrate NO and product N2O.

Materials and methods

Desulfovibrio vulgaris Hildenborough carrying the recombinant plasmid pJSP104 for the ~ 25-fold overproduction of the Hcp [22] was cultured anaerobically [23] and the Hcp was purified as described in [7]. Final preparations were 15–30 mg.mL–1 Hcp in 20 mM TrisHCl, pH 8.0 and 130 mM NaCl.

NO reduction activity was assayed in 1 mL liquid in 5 mL volume anaerobic bottles under helium atmosphere. The liquid contained 40 mM Heps buffer, pH 7.5, 1 mM benzyl viologen (BV), 5 mM sodium dithionite, ~1 µg Hcp. The reaction was started by injection of 100 µL of water saturated with NO scrubbed from traces of NO2 by bubbling through a 1 M solution of NaOH. The final concentration of NO in the reaction mixture was 212 µM. After a given reaction time the 5 mL bottle was vigorously shaken and a 1 mL gas sample was taken from the headspace and immediately injected in the gas chromatograph. Gas chromatography was done with a Fisons Instruments GC 8340 (Interscience, Breda, the Netherlands) following [24]. Briefly, 15 min runs were made with helium as the carrier gas in which N2O was detected at ~ 4.2 min with a 63Ni electron capture detector and NO was detected at ~ 11 min with a hot wire detector.

Concentrated (~ 200 µM) samples of oxidized and reduced Hcp for EPR spectroscopy were incubated at 20 °C with putative small-molecule reactants. The reduced Hcp was prepared by subsequent additions of small volumes of dithionite solution with EPR monitoring of the extend of reduction. NO was taken with a gas-tight 1 mL syringe from the headspace of an alkaline solution bubbled with 5% NO+ 95% He, and injected in the argon-filled head space of an anaerobic EPR tube containing 200 µL dithionite-reduced Hcp sample. Liquid and gas phase were equilibrated for 1 min with a small magnetic stirring bar inside the EPR tube driven by a ring of five stirring bars on the outside of the tube, giving a final concentration of ~ 100 µM NO (aq). After stirring, the bar was moved to the top of the EPR tube, the sample was frozen by immersion in liquid nitrogen, then disconnected from the closed tap of the argon/vacuum manifold, and the bar was taken out. Contrarily, N2O was added by bubbling the Hcp solution in a small anaerobic bottle with pure gas for 1 min giving a final concentration of 28.6 mM followed by transfer to anaerobic EPR tubes. CO was added by bubbling the Hcp solution with pure gas for 1 min to a final concentration of 1.07 mM. EPR spectroscopy was done with a Bruker ER 200D (Bruker Nederland BV, Leiderdorp, the Netherlands) and a Bruker EMXplus spectrometer using field modulation at 100 kHz, and with a home-built helium flow cooling system. The EPR data were analyzed with software as described in [25].

Results

Conversion of NO to N2O by D. vulgaris Hcp

The natural reduct partner of Hcp in D. vulgaris is not known; however, the hybrid cluster can exist in four stable oxidation states that differ by one electron [20], and a large structural change in the cluster, which is likely to be of catalytic relevance, is associated with the redox transition of lowest potential [12] which at pH 7.5 has an $E_m = -165$ mV [20]. Thus, for NO reduction to be exergonic, an artificial electron donor should have a potential below this value. Also, since the second prosthetic group of D. vulgaris Hcp is a regular [4Fe-4S]$^{(2:1+)}$ cluster, a one-electron donor is
called for. A common choice for enzyme assays is dithionite-reduced BV semiquinone with pH-independent $E_m = -374$ mV [26]. In practice, we encountered major problems of NO instability in optical and in electrochemical assays (not shown) presumably due to background reactions of NO with the BV radical and/or with the SO$_3^-$ radical, that is, the active redox agent in the dithionite system.

Only discrete off-line GC determination of substrate-product levels from headspace was found to give interpretable results (Fig. 2), which we present here as the first, be it semi-quantitative, detection of NO reductase activity from an Hcp that is not part of an operon-coded electron transfer chain.

After correction for a constant background level of N$_2$O in the absence of Hcp and correction for the disappearance of NO (without production of N$_2$O) in the absence of Hcp, we find an apparently Hcp catalyzed conversion of NO into N$_2$O which is linear in time and corresponds to an apparent specific activity of 130 nmol NO reduced min$^{-1}$ (mg Hcp)$^{-1}$. This value is some 18 times lower than the specific activity reported for E. coli Hcp presumably reflecting our use of a non-natural electron donor and/or inhibiting high NO concentrations. To put things in perspective: the membrane bound Ralstonia eutropha NO reductase whose natural e-donor is probably a quinol, exhibits, after purification, a specific activity in the range of 160–2270 nmol min$^{-1}$ mg$^{-1}$ depending on which reduced quinone is used [27]. Overall the relatively low activity is probably the result of a far from optimal assay and thus should be taken as a qualitative proof that D. vulgaris Hcp carries NO reductase activity.

**Reaction with NO affords a redox-active protein-dinitrosyl iron complex**

Incubation of oxidized or dithionite-reduced D. vulgaris Hcp at high concentration with dissolved NO at substoichiometric concentration affords small EPR signals around $g \approx 2$ characteristic for dinitrosyl iron complexes [28–33] on top of the signal of the oxidized or the fully reduced hybrid cluster. The EPR spectrum of oxidized (as isolated) Hcp consists of two signals (grey bars in Fig. 3A): a complex $S = 9/2$ signal, with predominant features at low field, which is difficult to quantitate, and a minor $S = 1/2$ signal just below $g = 2$, which integrates to ~0.1 spin per Hcp [20]. Upon incubation with NO, the signals somewhat decrease in intensity and a small signal near $g \approx 2$ appears, which turns out to be heavily saturated under the conditions used to monitor the original hybrid cluster signals. When recorded at higher temperature and lower nonsaturating power (Fig. 3B), the signal is found to have a considerable intensity corresponding to twice that of the original $S = 1/2$ hybrid cluster signal, that is ~0.2 spins per Hcp. The signal is strongly reminiscent of the EPR ($g_L \approx 2.04, g_t \approx 2.015$) from frozen solutions of model compounds of the type [Fe(NO)$_2$XY], where X and Y are anionic ligands [29]. In a seminal study on these type of compounds the iron in the Fe(NO)$_2$ unit was found to be best described as Fe(I), d$^7$ with $S = 1/2$ [28]. Similar low-temperature EPR signals have previously been reported for bacterioferritin [30] and for ferritin [31] after reaction with NO. Addition of excess, 20 mM, of the slow reductant dithionite, and incubation for 5 min leads to ~90% disappearance of the dinitrosyliron signal consistent with enzymic turnover producing N$_2$O.

Reduced Hcp exhibits EPR of a single $S = 1/2$ system only (Fig. 3C), which is approximately stoichiometric with protein concentration when based on a molecular mass of 60 kDa [1,6]. Reaction with NO leads to reduction in the signal amplitude concomitant with the appearance of a new signal in the $g \approx 2$ region. Also when run under nonsaturating conditions (Fig. 3D), this signal is found to integrate to ~0.2 spins per original $S = 1/2$ signal, that is per Hcp. The spectrum is very similar to that reported for the two-electron reduced form of [Fe(NO)$_2$X$_3$], in which X is glutathione, in which the iron is formally Fe(-I), d$^9$, with $S = 1/2$ [33]. This spectrum is of axial symmetry, and the low-field line is from some unreduced [Fe(NO)$_2$XY].

![Fig. 2. NO reductase activity of Desulfovibrio vulgaris Hcp. The actually measured NO consumption (green), the NO consumption corrected for nonenzymatic consumption (red), and the N$_2$O generation corrected for constant N$_2$O background (blue) are plotted. Hcp, 20 nm, was in 40 mM Hepes buffer, pH 7.5, with 1 mM BV and 5 mM sodium dithionite. The reaction was started by addition of 212 μM NO (aq).](image-url)
There is also a weak $S = 3/2$ signal with $g \approx 4$ (asterisk in Fig. 3C) that can be ascribed to mononitrosyl Fe(II)-NO complex \(^{34}\). Based on an $S = 3/2$ simulation, its integrated intensity is only 0.005 spins per Hcp, therefore it is probably the result of the reaction of a trace of junk iron (aspecifically bound iron) with NO and not an enzymatic intermediate. It is concluded that when $\sim 200 \mu M$ Hcp is incubated with $\sim 100 \mu M$ NO, the substrate is nearly quantitatively ($\sim 80 \mu M$) found back as a dinitrosyl iron complex either in oxidized or in reduced Hcp, and there is no significant formation of mononitrosyl complex. A structural similarity has been noted between the hybrid cluster and the nickel-iron-sulfur cluster of \textit{Carboxydothermus hydrogenoformans} carbon monoxide dehydrogenase with concomitant sequence homology \(^{11}\). We therefore checked for effects of CO incubation on the EPR of oxidized or reduced \textit{D. vulgaris} Hcp. Maximal solubility of CO in water at 20 °C is 1.07 mM. No EPR spectral changes were found both for oxidized and for reduced Hcp.

**Association complex of the reduced hybrid cluster with N$_2$O**

Since N$_2$O is the product of the enzyme-catalyzed reaction, one would expect the Hcp to exhibit low affinity for this leaving group. However, N$_2$O has significant reactivity versus water and its solubility at 20 °C amounts to 28.6 mM. We therefore tested for changes in the EPR of Hcp upon flushing with pure N$_2$O. The spectrum of oxidized Hcp was found to be unaffected (not shown) but the spectrum of reduced Hcp showed significant changes (Fig. 4).

In the characteristic spectrum of the reduced hybrid cluster, we found shifts in at least two of its $g$ values from 2.010, 1.823, 1.34 to 1.982, 1.823, 1.29, with all $g$ values now below the free-electron value. The total

**Fig. 3.** EPR spectra of unreacted (blue lines) and dinitrosyl iron complex containing (red lines) \textit{Desulfovibrio vulgaris} Hcp. (A) Overview spectra of oxidized Hcp showing the major $S = 9/2$ and the minor $S = 1/2$ signals from the hybrid cluster; the thick arrow indicates a minor change upon NO incubation, which (B) gains relative intensity when measured at lower microwave power. (C) Overview spectra of reduced Hcp showing the very anisotropic $S = 1/2$ signal from the hybrid cluster; the thick arrow points to a small spectral change, which (D) gains relative intensity when measured at lower power. The black traces are simulations based on the indicated $g$ values. The weak signal in the red trace of panel C indicated with an asterisk, just next to the $g = 4.3$ signal from junk iron, is ascribed to Fe(II)-NO complex with $S = 3/2$ \(^{34}\). The enzyme, 180 μM Hcp in 20 mM TrisHCl, pH 8.0, 130 mM NaCl, was oxidized (as isolated) or stepwise fully reduced by incubation with − 2 mM dithionite for 5 min. The substrate was added as described under Methods to a final concentration of − 100 μM NO. EPR conditions of microwave frequency, microwave power, modulation amplitude, temperature: (A) 9405 MHz, 32 mWatt, 10 gauss, 16 K; (B) 9414 MHz, 0.13 mWatt, 6 gauss, 36 K; (C) 9428 MHz, 80 mWatt, 12.5 gauss, 17 K; (D) 9429 MHz, 0.20 mWatt, 6.3 gauss, 18.5 K.

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integrated intensity was not significantly affected so the cluster remains fully reduced.

The observed changes are sufficiently pronounced to suggest that N2O directly interacts with the reduced hybrid cluster. Since there are drastic structural changes between the reduced and the oxidized cluster, our observations may suggest that dissociation of the product N2O only occurs after re-reduction of the hybrid cluster.

Discussion

A basic paradigm of enzymology is the initial identification of a biological activity followed by the subsequent isolation and characterization of the responsible protein system. Occasionally this order of things is reversed when a novel protein is found and its catalytic function is only later identified. A rather extreme case in point is the metalloprotein known as the Hcp, which we accidentally purified some three decades ago, and whose activity only jerkily transpired in recent years while in the intervening time the protein has been extensively scrutinized spectroscopically as well as crystallographically. Furthermore, 30 years of intensive research fell short of providing yet a single clue as to the mechanism (or mechanisms) of action underlying the activity (or activities) of Hcp. The present work is an attempt to create a first ingress into mechanistic studies of Hcp.

Our starting points have been (a) the assumption that activity is located at the hybrid cluster; (b) the observation that, for example, optical properties of Hcp are rather featureless [7] but EPR properties are unique and detailed [20] and therefore should provide a good monitor of reaction intermediates; (c) the fact that the nitric oxide/nitrous oxide couple forms a substrate/product pair for at least one particular Hcp, the E. coli one [4], that is, the enzyme catalyzes the overall (i.e., ignoring intermediates) reaction

$$2\text{NO} + 2\text{[H]} \leftrightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$$

Behind this simple equation, a number of basic mechanistic challenges is waiting to be addressed. Is the product N2O or rather H2N2O2/HN2O2? Crystallographic inspection has identified both hydrophobic channels and a solvent channel giving access to the hybrid cluster [10]. The latter may be the entry of protons, but it may also be the exit of an anion. Furthermore, the source of the reducing equivalents is only known for E. coli Hcp [2]. And finally, in a different class of NO reductases (heme-copper oxidases, HCuO, superfamily) NO has been shown to give rise to substrate inhibition [35]. Furthermore: what is the – initial – ES complex? In analysis of steady-state kinetics of HCuO-type NO reductases, the reaction is usually written as (Fig 5) in which the first two (presumed reversible) reactions represent binding to one and the same or to two different Fe ions, and in which $K_1$ and $K_2$ have been reported to be of comparable magnitude [35,36]. The corresponding rate equation is [35]

$$v = \frac{k_{cat}[E]_r}{(1 + K_2(1/[\text{NO}] + K_1/[\text{NO}]^2))}$$

However, when Wang et al. [4] fit their steady-state kinetic data for E. coli Hcp to this equation they find $K_1 = 0$, which implies an infinite, or at least an extremely high affinity of Hcp for the first NO substrate. In other words, in the presence of substoichiometric amounts all NO should be bound to one specific binding site on the protein with the second NO binding site unoccupied, that is, a stable mononitrosyl intermediate should be formed. Furthermore, Seth et al. [5] claim that auto-S-nitrosylation of E. coli Hcp is via formation of an Fe(II)-NO complex.

Both predictions do not appear to be fully compatible with our observation of metastable dinitrosyliron complexes as the first oxidized and reduced ES

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**Fig. 4.** Changes in the EPR spectrum of reduced hybrid cluster in *Desulfovibrio vulgaris* Hcp upon incubation with N2O. The enzyme, 215 μm in 20 mTrisHCl, pH 8.0, 130 mNaCl, was flushed with pure N2O gas for 1 min. The inset is a zoom in on the $g_z$ area. EPR conditions: microwave frequency, 9225 MHz, microwave power, 80 mwatt, modulation amplitude, 10 gauss, temperature, 16 K.

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**Fig. 5.** Steady-state kinetics proposed for HCuO-type NO reductases [35,36].
complexes in combination with reactivity observed as partial disappearance of the EPR signal of the hybrid cluster. The $K_1 \approx 0$ fitting result of Wang et al. [4] leads them to conclude that the steady-state kinetics is a simple Michaelis–Menten pattern with $K_2 \equiv K_M$, however, the subsequently calculated catalytic efficiency, $k_{cat}/K_M$, then amounts to $2.4 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, which is an unlikely number as it would be an all-time record in enzymology, and, in fact, would transgress the limit dictated by diffusion limitation. Indeed, when inspecting their kinetic traces, we find that the presented fit is not unique and that equally good fits can be obtained with $K_1 \gg K_2$, which can be seen as a basic form of cooperativity in dinitrosyl formation. We conclude that the NO reductase kinetics for purified E. coli Hcp [4] is open to re-interpretation. With regard to the conclusion by Seth et al. that purified E. coli Hcp, of unspecified redox state forms an Fe (II)-NO complex, of unspecified structure, followed by S-nitrosylation of a (hitherto) cluster-coordinating cysteine, we note that the occurrence of a dinitrosyliron complex in Hcp may obviate the need for auto-S-nitrosylation as a preparative step for trans-S-nitrosylation to other proteins because dinitrosyliron complexes are known to be ready donors of NO [32].

Which iron in the hybrid cluster (Fig. 1C,D) is the binding site for the two NOs? In the absence of experimental information to build on, we make the following cautious observations to be tested in future experiments. Fe5 and Fe6 form a classical Fe$_2$S$_2$ diamond and, although its external ligation deviates from the classical 4Cys pattern, it is hard to imagine how one of the iron's in this substructure could bind 2 NO without decomposition of the diamond. In the enzyme without substrate upon cluster reduction Fe7 and Fe8 both undergo a drastic change in coordination and Fe8 moves over a distance of 2.1 Å [12]. This coordinative flexibility would seem to suggest that either of them could act as a putative binding site for the 2 NO. The question how the dinitrosyl iron would interact electronically and magnetically with the other iron in the cluster should be addressed in future magnetic-spectroscopic studies.

Taken together, our data suggest that Hep may act as a key mechanistic bifurcation of pathways, a signal-transducing one and a metabolic one, interrelated by the redox status of the enzyme as outlined in the following scheme, in which HC stands for hybrid cluster (Fig 6).

In its incompleteness, this simple scheme also suggests a number of static and dynamic new experiments to further our knowledge of Hep in terms of reaction mechanism(s). Examples would be: cryo crystallography of oxidized Hep after anaerobic incubation at ambient temperature of a crystal under NO gas to determine the molecular binding mode(s) of NO to the hybrid cluster; monitoring trans-nitrosylation by the E. coli Hcp/Hcr system as a function of NADH/NAD$^+$ ratio to probe redox regulation of the proposed bifurcation; freeze-quench kinetics of oxidized or reduced Hep with NO-saturated buffer to check for short-lived mononitrosyl intermediates.

Finally, with reference to the structures in Fig. 1, it seems to us that the crystallographically documented drastic structural changes that occur in the hybrid cluster, when it goes from ‘4+’ to ‘3+’, are really a bit too much to ask for as the effect of a single-electron reduction only. Clearly, we are missing something here. Perhaps the ‘as isolated’ Hep, whether isolated aerobically (5+) or anaerobically (4+) is a resting, un-activated form of the enzyme, and therefore reduction plus re-oxidation would be required to obtain the active oxidized form (which has not yet been crystallized). In this respect, our observation that the product N$_2$O binds to the reduced, and not to the oxidized enzyme would suggest the following picture: if N$_2$O or H$_2$N$_2$O$_2$ would bind (in)to the reduced hybrid cluster, the result could rather look like the extant structure of the oxidized cluster. It is clear

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**Fig. 6.** Proposal for a redox-dependent bifurcation scheme of Hcp activities.
that further studies are required both in the steady-state and in the pre-steady-state kinetics of this after 30 years still highly enigmatic protein.

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**Author contributions**

WRH conceived the study, designed and performed the analysis of many batches of Hcp s in Wageningen.

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