Iron-sulphur clusters: agents of electron transfer and storage, and direct participants in enzymic reactions

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Introduction: a historical conference

In the spring of 1932, when I was labouring in the examinations of the ‘Abiturium’ at the Gymnasium of my home town Heidelberg — when the world seemed to open up before me — I was not aware that just in those days, about a mile further down the Neckar river, there was being held at the then newly founded Kaiser-Wilhelm- (now Max Planck-) Institute for Medical Research, a meeting entitled ‘Vorträge und Demonstrationen über Probleme und Grundlagen biologischer Oxydationsvorgänge’, which brought together the world’s foremost brains in the field of biological oxidations. It is always with great respect that I look at the page of the pig-leather-bound book where guests and visitors of that Institute signed in (Fig. 1). I did not know any of the participants, neither did I have the faintest notion that the field of biological oxidations would be the field of my main interest and endeavour for many years or that I would some day have the honour of lecturing in memory of David Keilin, the second signatory on that historical page.

While it is probably true that today, because of the enormous progress in the last few decades, every graduate student of biochemistry knows more about the theme under discussion at that meeting than the participants of those days, it is equally true that, if it had not been for the thoughts and endeavours of those men, we would not have the insights of which we pride ourselves today. Today, I wish I knew that they were talking about! Surely about cytochromes, flavoprotein and oxidation-reduction potentials — which featured highly in those days. The idea of a ‘respiratory chain’ was conceived in those years and the contributions of David Keilin to this area are known to all of us.

It was just in Keilin’s last years of life that the respiratory chain underwent its greatest expansion with the advent of ubiquinone, copper (copper had originally been found to be associated with cytochrome c oxidase by Keilin and Hartree but approaches to prove its significance were not available at that time) and non-haem iron — as we now know largely Fe-S proteins.

Iron-sulphur proteins in the respiratory chain

While I am referring here to Fe-S proteins, we must realize that today our original simple ideas of Fe-S proteins in the respiratory chain have already become outdated. Until recently we knew of [2Fe-2S] and [4Fe-4S] clusters, we knew that the latter could occur in three different oxidation states, with nature utilizing either the lower redox couple, or, more rarely, the higher couple in any given Fe-S protein, and we tried to understand the structure and function of Fe-S proteins of the respiratory chain in such terms. It remained an enigma, though, why there should be so many of these proteins, more individual representatives than of any other known component of the respiratory chain.

As so often in research, answers are not forthcoming from direct frontal attack on a problem, but in a rather roundabout way from a different direction. Thus, for example, the Fe-S proteins of the nitrogenase components or in the various hydrogenases may have much to contribute to what we would like to know about Fe-S proteins of the respiratory chain; but we don’t even have to go that far from the realm of the respiratory chain. There is an enzyme of the tricarboxylic acid cycle, which nobody would have thought to have anything to do with iron-sulphur 15 years ago, namely aconitase, which is, nevertheless, an Fe-S protein (Kennedy et al., 1972; Ruzicka & Beinert, 1978); and this protein has taught us that Fe-S clusters in proteins can be unexpectedly malleable, may shed to the medium or avidly incorporate from it an extra iron ion (Kent et al., 1982; Kennedy et al., 1983; Emptage et al., 1983a), may exchange their labile sulphide within minutes with sulphide in solution (Kennedy et al., 1984a) and, correspondingly, the protein surrounding Fe-S clusters may accommodate the resulting structures involved in such interconversions (cf. Kennedy et al., 1984a).
Fig. 1. Copy of a page from the guest-book of the Kaiser-Wilhelm- (now Max Planck-) Institut für medizinische Forschung, Heidelberg: the participants of a conference on biological oxidations.

From Max-Planck-Gesellschaft, Berichte und Mitteilungen I/1980, with the permission of Professor Theodor Wieland.
Moreover, we have learned from this enzyme the entirely unexpected lesson that Fe-S clusters are not, in all instances, agents of electron (e-) transfer and temporary storage, but may, with very specific parts of their structure, enter into enzyme-substrate reactions (Emptage et al., 1983b). These developments, which to me seem to add a new dimension to Fe-S protein research, and consequently also to our ideas of their functions in respiratory chains, will be the theme of my lecture today.

Fe-S clusters in e- transfer and storage

During the 25 years between 1950 and 1975 our picture of the respiratory chain of mammalian mitochondria changed dramatically (Fig. 2), most conspicuous being the multitude of Fe-S components that have been added. Why should NADH dehydrogenase require at least four Fe-S clusters and, according to Fe and S2- analysis, possibly even more, which have not yet been clearly identified by e.p.r. spectroscopy? (Ohnishi et al., 1985). From the example of the simplest Fe-S proteins, namely the ferredoxins of plants and microbes, we know that Fe-S proteins are efficient agents of e-transfer (Lovenberg, 1973), largely in the region of low oxidation-reduction potentials. The finding of a variety of Fe-S clusters occurring in complex e-transfer systems such as those of xanthine oxidase, succinate and NADH dehydrogenases, nitrite and sulphite reductases, or the nitrogenase components has fostered the notion of a temporary storage function, of an e-exchange bank, so to speak, for some of the Fe-S clusters. Such a function would facilitate conversions from one-e- to two-e-transfer in complex systems and could allow withdrawal of two e- consecutively at the same potential level. Although a number of systems have been investigated which point to such a function (Olsen et al., 1974a, b; Kamin & Lambeth, 1982) and some schemes have been proposed (Kamin & Lambeth, 1982; Hemmerich, 1977), it has been difficult to provide unambiguous proof for such a function in intramolecular e-transfers in view of the rapidity with which intramolecular e-exchanges usually take place. One of the experimentally most yielding examples was that of the di- and tri-methylamine dehydrogenases from methylotrophic bacteria (Steenkamp & Beinert, 1982a, b). These enzymes possess a single flavin (FMN) and [4Fe-4S] cluster per active site. They receive 2e- from the substrate. According to spectrophotometry in the liquid and frozen state, the FMN is reduced to FMNH, within a few milliseconds after addition of substrate (Steenkamp & Beinert, 1982a, b) and then follows a slower reaction in which spectrophotometry registers a flavin radical, while e.p.r. shows the unambiguous signal of a triplet state. No significant signal of a reduced Fe-S cluster is obtained by e.p.r.
Titration with substrate shows that, despite the capacity of the system to accommodate 3e\(^-\), only two e\(^-\) are accepted by the enzyme from substrate. Thus, since only two e\(^-\) are in the system, since FMN and the Fe-S cluster are the only prosthetic groups (an X-ray structure analysis of the enzyme is available; Matthews et al., 1984) and e.p.r. shows two interacting parallel spins, the triplet state must arise by single e\(^-\) transfer from FMNH\(_2\) to the Fe-cluster with formation of FMNH and [4Fe-4S]\(^{2+}\) (Fig. 3), the two interacting paramagnetic partners. The disparate rates of the initial two-e\(^-\) transfer from substrate to flavin and of the ensuing one-e\(^-\) transfer from FMNH\(_2\) to [4Fe-4S]\(^{2+}\), and the opportunity to observe this second e\(^-\) transfer reaction independently by e.p.r. through the appearance of the triplet signal, have made it possible in this case to clearly resolve the events. The very fact that a triplet state arises, showing an unusually strong half-field e.p.r. signal and thus implying close proximity of the interacting paramagnets, leaves no doubt that e\(^-\) transfer is intramolecular. The enzyme in the triplet state with one e\(^-\) in the FMNH\(_2\) and one in the Fe-S cluster rapidly transfers these electrons to a flavoprotein with properties similar to the e\(^-\) transfer flavoprotein (ETF) in the \(\beta\)-oxidation system of fatty acids (Crane & Beinert, 1956). This flavoprotein presumably is the natural e\(^-\) acceptor of the di- and tri-methylamine dehydrogenases. It forms a very stable anionic semiquinone and has never been observed in its fully reduced state and thus requires single e\(^-\) transfer. Unfortunately, it has not been possible yet to complete this example by ascertaining exactly in what sequence the e\(^-\) transfer to this acceptor occurs, but the initial e\(^-\)pair splitting function of the flavin-Fe-S combination in preparation for a one-e\(^-\) transfer has become very obvious in this instance.

Fe-S clusters in non-oxidative enzymes: participation in enzyme-substrate reactions

Aconitase as an Fe-S protein. The enzyme aconitase has long been known to require iron for optimal function (Morrison, 1954; Villafranca & Mildvan, 1971; Gawron et al., 1974). In fact, when highly purified (\(\geq 95\%\)), the enzyme loses almost all of its activity. This can, however, be regained by an activation process, for which traditionally ferrous iron and some thiol compounds have been used. The absolute requirement for Fe became controversial, when it was observed that mere reduction of the enzyme could restore up to 75\% of its maximal activity (Kennedy et al., 1983). Aconitase has an e.p.r. signal in its oxidized state as it is obtained on purification, indicative of an Fe-S cluster (Kent et al., 1982). As long as only [2Fe-2S] and [4Fe-4S] clusters were known in Fe-S proteins, it was thought (Kent et al., 1982) that this signal originated from a [4Fe-4S]\(^{2+}\) cluster in its highest oxidation state, analogous to that of the bacterial 'high-potential Fe-S proteins' (HiPIP; Palmer et al., 1967). However, the newly developed technique of cluster extrusion indicated that a [2Fe-2S] cluster was present (Kurtz et al., 1979). Then, in 1980, strong evidence was obtained from Mössbauer and e.p.r. spectroscopy of other Fe-S proteins (Emptage et al., 1980) that 3Fe clusters must exist and these clusters showed e.p.r. signals almost identical in shape and \(g\)-value to that of aconitase. It then became clear from a synopsis of the results of Mössbauer and e.p.r. spectroscopies that aconitase also had a 3Fe cluster. Since, at that time, it was generally believed that, in proteins at least, Fe and S\(^{2-}\) would have to occur in a 1:1 stoichiometry in clusters, it was assumed that aconitase had a [3Fe-3S] cluster as it had been assumed for other similar proteins (Emptage et al., 1980). A flat six-membered ring structure was proposed from X-ray studies on ferredoxin (Fd) I of Azotobacter vinelandii for such a cluster type (Ghosh et al., 1982).

Incorporation of Fe into aconitase on activation: formation of a 4Fe from a 3Fe cluster. In a Mössbauer investigation of aconitase reduced by dithionite, spectra were observed which indicated the presence of reduced 4Fe clusters ([4Fe-4S]\(^{2+}\)) in the samples (Kent et al., 1982). This was verified by e.p.r. through the finding of an e.p.r. signal typical of reduced 4Fe Fd's. Since no 4Fe clusters had been seen by Mössbauer spectroscopy in the oxidized state, these 4Fe clusters obviously arose on reduction of aconitase. When at pH 8.5 and in the presence of the mediator methylviologen the yield of 4Fe clusters on reduction could be raised to \(\sim 80\%\), it became clear that we were dealing here with a cluster interconversion from a 3Fe to a 4Fe cluster. We then suspected that this
interconversion may be nothing else than what we had thus far called activation of the enzyme facilitated by the addition of extra Fe (Kennedy et al., 1983). It was shown by the use of radioactive iron that one Fe ion was incorporated per 3Fe cluster of the inactive enzyme to form the 4Fe cluster of active aconitase and this incorporated Fe was again specifically removed on oxidation (Emptage et al., 1983a; Kennedy et al., 1983). This was verified by Mössbauer spectroscopy, which showed that the enzyme when activated in the usual way by Fe and thiol (Kent et al., 1982) (not with dithionite) did not yield the $^{1+}$ but the $^{2+}$ form of a $[4Fe-4S]$ cluster, which was the active form of the enzyme (Fig. 4; this cluster obviously had been reduced to its $^{1+}$ form in the above-mentioned experiments using dithionite reduction). Activation of the enzyme in the absence of Fe was then due to internal rearrangement of cluster structures such that ultimately 25% of the 3Fe clusters originally present contributed their iron to the completion of 4Fe clusters in the remaining 75%; hence the observation of only 75% of maximal activity after activation with reductant only.

In these interconversions $S^{2-}$ was never required and it could be shown by improved analytical methods (Beinert, 1983; Beinert et al., 1983; Ryden et al., 1984) that inactive aconitase, as obtained on routine purification, contained a $[3Fe-4S]$ cluster, the first clearly documented deviation from the generally assumed 1:1 stoichiometry for Fe–S clusters in proteins.

Mössbauer spectroscopy of active 4Fe aconitase: the site of substrate interaction. The circumstances just described made it possible, of course, to label a specific site in the active enzyme, e.g. with $^{57}$Fe for Mössbauer spectroscopy, or $^{55}$Fe or $^{59}$Fe for radiochemistry. Scrambling of Fe between the four sites of the cluster did not occur significantly at neutral pH over periods of a few hours. However, when the enzyme was exposed to pH 9.5 in the presence of oxygen and then neutralized and activated anaerobically with $^{57}$Fe, the label was distributed over all sites with formation of the 4Fe form. Oxidation then yielded the labelled 3Fe form and subsequent reactivation of this form with $^{56}$Fe led to the active enzyme with $^{57}$Fe in the other three sites.

Previous e.p.r. experiments on the 3Fe cluster, as it exists in the inactive enzyme obtained on purification, had shown that addition of substrate has only minor effects on its e.p.r. spectrum, no more than a number of other unrelated anions do. However, the incorporation of $^{57}$Fe into specific sites of the cluster of the active form, as just described, made it now possible to look by Mössbauer spectroscopy for substrate effects on these sites (Emptage et al., 1983b). There was indeed a dramatic effect of substrate addition on the Fe ($^{57}$Fe for Mössbauer spectroscopy) ion, in the site into which it is uniquely incorporated on activation, which we may call Fe$_{4}$, whereas the Fe ions in the other sites (Fe$_{3}$), when labelled, were not significantly affected. The isomeric shift, $\delta$, of Fe$_{4}$ almost doubled, from 0.46 to 0.84 and 0.89 mm/s (two species arise), and the quadrupole splitting, $\Delta E_Q$, changed from 0.83 to 1.26 and 1.83 mm/s. Values of this magnitude have never been seen with tetrahedrally coordinated Fe in [4Fe–4S] clusters at any oxidation state. Since, according to Mössbauer spectroscopy in a strong magnetic field, spin coupling among the four Fe ions of the cluster was maintained, i.e. all four Fe ions were still part of the cluster, it was proposed that the coordination state of Fe$_{4}$, changed from 4 to 5 or higher (Emptage et al., 1983b). Simultaneously, an increase in electron density at this ion was indicated by the high isomeric shift and quadrupole splitting typical of high-spin Fe$^{2+}$. These effects have led us to the concept that Fe$_{4}$, the fourth Fe ion introduced on activation, is the site of substrate binding and participates in the interconversion of tricarboxylic acids catalysed by aconitase.

The group on the substrate binding to the Fe–S cluster. Since Mössbauer spectroscopy specifically looks at $^{57}$Fe ions only, it now required a different technique to identify the group(s) of the substrate which were interacting with the Fe$_{3}$ ion. In activated aconitase the spins of the Fe ions are coupled such that a diamagnetic state results. Thus...
there is no e.p.r. signal from this state. Fortunately, however, the reduced cluster, [4Fe-4S]^{+} \cdot has a Fd-type e.p.r. signal, and, to the best of our knowledge, aconitase has about 30% of its maximal activity in this state (Emptage et al., 1983\textsuperscript{a}). All g-values of this [4Fe-4S]+ form undergo a sizeable shift when substrate is added. The groups of the substrate most likely to be involved in binding to iron are the hydroxyl in position 3 or 2 of citrate, or isocitrate, respectively, or the carboxyl groups. We, therefore, tested whether \textsuperscript{17}O-labelled substrates would produce any broadening of the e.p.r. resonances through unresolved hyperfine interactions with the spin system of the cluster. The three carboxyl groups were labelled specifically by exchange with H\textsubscript{2}\textsuperscript{17}O (water enriched 38% in \textsuperscript{17}O) at high temperature and low pH, while the hydroxyl of citrate or isocitrate rapidly exchanges with H\textsubscript{2}\textsuperscript{17}O in the presence of active aconitase (Rose & O'Connell, 1967). There was no broadening observed with carboxyl-labelled substrate, but there was significant broadening of all resonances in the presence of H\textsubscript{2}\textsuperscript{17}O and substrate. Thus, either H\textsubscript{2}O or the hydroxyl of substrate is bound to Fe\textsubscript{a} in active aconitase.

We used two approaches in attempts to decide between these two possibilities. First, trans-aconitate is known as a competitive inhibitor, not a substrate, of the enzyme. Addition of trans-aconitate produced a shift of g-values of the e.p.r. signal of the [4Fe-4S]+ form similar to but not identical with that observed with the substrates (Emptage et al., 1983\textsuperscript{b}). Again, there was significant broadening of the resonances when H\textsubscript{2}\textsuperscript{17}O was substituted for H\textsubscript{2}O. Since the hydration reaction of the double bond does not proceed with trans-aconitate, this seemed a strong argument in favour of H\textsubscript{2}O being bound to Fe\textsubscript{a} during substrate binding in the enzyme system of the cluster. The three carboxyl groups were labelled specifically by exchange with H\textsubscript{2}\textsuperscript{17}O (water enriched 38% in \textsuperscript{17}O) at high temperature and low pH, while the hydroxyl of citrate or isocitrate rapidly exchanges with H\textsubscript{2}\textsuperscript{17}O in the presence of active aconitase (Rose & O'Connell, 1967). There was no broadening observed with carboxyl-labelled substrate, but there was significant broadening of all resonances in the presence of H\textsubscript{2}\textsuperscript{17}O and substrate. Thus, either H\textsubscript{2}O or the hydroxyl of substrate is bound to Fe\textsubscript{a} in active aconitase.

We, therefore, tried to freeze-quench samples in cups suitable for Mössbauer spectroscopy. By mixing samples at 0°C we were able to observe significant, time-dependent differences between samples to which citrate, isocitrate or aconitate were added (Kent et al., 1985). Obviously the complexes formed between each substrate and the enzyme traverse different and discrete states, which are expressed in the Mössbauer spectra of Fe\textsubscript{a} in the cluster. The changes occur most rapidly with isocitrate, suggesting that the majority of the enzyme molecules have the proper conformation to accommodate isocitrate. This would be in agreement with the fact long known from conventional kinetic observations that, with isocitrate as substrate, uniformly higher rates are observed than with citrate.

Some observations on initial events in the aconitase reaction. The rate of the aconitase reaction is just at the border of what one may hope to resolve into individual steps by rapid mixing and freezing techniques. We have, therefore, tried to freeze-quench samples in cups suitable for Mössbauer spectroscopy. By mixing samples at 0°C we were able to observe significant, time-dependent differences between samples to which citrate, isocitrate or aconitate were added (Kent et al., 1985). Obviously the complexes formed between each substrate and the enzyme traverse different and discrete states, which are expressed in the Mössbauer spectra of Fe\textsubscript{a} in the cluster. The changes occur most rapidly with isocitrate, suggesting that the majority of the enzyme molecules have the proper conformation to accommodate isocitrate. This would be in agreement with the fact long known from conventional kinetic observations that, with isocitrate as substrate, uniformly higher rates are observed than with citrate.

### Conclusions and outlook

To summarize our conclusions: inactive aconitase, as we prepare it in the laboratory, has a [3Fe-4S] cluster. On activation this cluster is converted to a [4Fe-4S]\textsuperscript{+} cluster which corresponds to the enzyme that occurs in vivo. From this a paramagnetic-reduced form [4Fe-4S]\textsuperscript{+} with diminished activity can be prepared by strong reductants at pH = 8.5. Both the 2 + and 1 + forms can be shown by Mössbauer or e.p.r. spectroscopy to respond strongly to substrate addition. In the course of the aconitase reaction the single Fe ion incorporated on activation expands its coordination sphere and acquires electron density (according to Mössbauer spectroscopy), while the hydroxyl of citrate or isocitrate and in addition H\textsubscript{2}O from the medium are bound to this Fe (according to e.p.r.). At early reaction times some discrete states of the enzyme substrate complexes can be resolved by Mössbauer spectroscopy, which differ for the different substrates and which develop most rapidly with isocitrate.

I have spent disproportionately more time on this last aspect of Fe-S cluster function, namely that of direct participation in an enzyme-substrate reaction, because it represents an entirely new aspect of Fe-S cluster chemistry and function, whereas electron transport and exchange are by now well-recognized functions of Fe-S clusters.
Coming back to the theme alluded to initially, namely the mammalian respiratory chain: we must take another close and critical look at the multitude and variety of Fe-S clusters apparently involved in this system and we must consider the possibility that some of these clusters might undergo reactions as we have shown them to occur with aconitase. This is not to say that we should expect to find dehydration-hydration reactions, but more generally, cluster–substrate (or other group) interactions, development of localized valence states and polarization effects. The same considerations as for the respiratory chain would, of course, equally apply to other complex systems involving Fe-S clusters such as, e.g., the related photosynthetic electron-transfer systems, nitrogenase or hydrogenases.

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