

Rapid report

# Proton transfer in *Azotobacter vinelandii* ferredoxin I: entatic Lys84 operates as elastic counterbalance for the proton-carrying Asp15

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## Abstract

In ferredoxin I from *Azotobacter vinelandii*, the reduction of a [3Fe-4S] iron-sulphur cluster is coupled with the protonation of the  $\mu_2$ S sulphur atom that is approx. 6 Å away from the protein boundary. The recent study of the site-specific mutants of ferredoxin I led to the conclusion that a particular surface aspartic residue (Asp15) is solely responsible for the proton transfer to the  $\mu_2$ S atom by ‘rapid penetrative excursions’ (K. Chen, J. Hirst, R. Camba, C.A. Bonagura, C.D. Stout, B.K. Burgess, F.A. Armstrong, Nature 405 (2000) 814–817). In the same paper it has been reported that the replacement of Asp15 by glutamate led to the blockage of the enzyme, although glutamate, with its longer and more flexible side chain, should apparently do even better as a mobile proton carrier than aspartate. We tackled this puzzling incompetence of Glu15 by molecular dynamics simulations. It was revealed that the conformational alterations of Asp15 are energetically balanced by the straining of the nearby Lys84 side chain in wild-type ferredoxin I but not in the Asp15 → Glu mutant. Lys84 in ferredoxin I of *A. vinelandii* seems to represent the first case where the strained (entatic) conformation of a particular amino acid side chain could be directly identified in the ground state of an enzyme and assigned to a distinct mechanism of energy balance during the catalytic transition. © 2001 Elsevier Science B.V. All rights reserved.

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Intraprotein proton transfer plays a key role in bioenergetics and is a subject of intense studies. Chen and co-workers have recently introduced ferredoxin I from *Azotobacter vinelandii* as a new, useful model system for studying proton transfer in proteins [1]. Although ferredoxin I is not a genuine proton pump, the reduction of its [3Fe-4S] iron-sulphur cluster is coupled with the protonation of the  $\mu_2$ S sulphur atom that is approx. 6 Å away from the

protein-water boundary [1,2]. The analysis of the site-specific mutants of ferredoxin I has revealed that only the replacement of a particular surface aspartic residue (Asp15), and not of the other, potentially proton-transporting residues in the vicinity, blocked the enzyme turnover [1]. Correspondingly, it was concluded that Asp15 is solely responsible for the proton transfer to the  $\mu_2$ S atom [1]. Although the minimal distance between the carboxy group of Asp15 and the  $\mu_2$ S atom is 4.4 Å in the crystal structure of the oxidised enzyme, the molecular dynamics simulations demonstrated that the protonated Asp15 side chain could reach the [3Fe-4S] cluster with a

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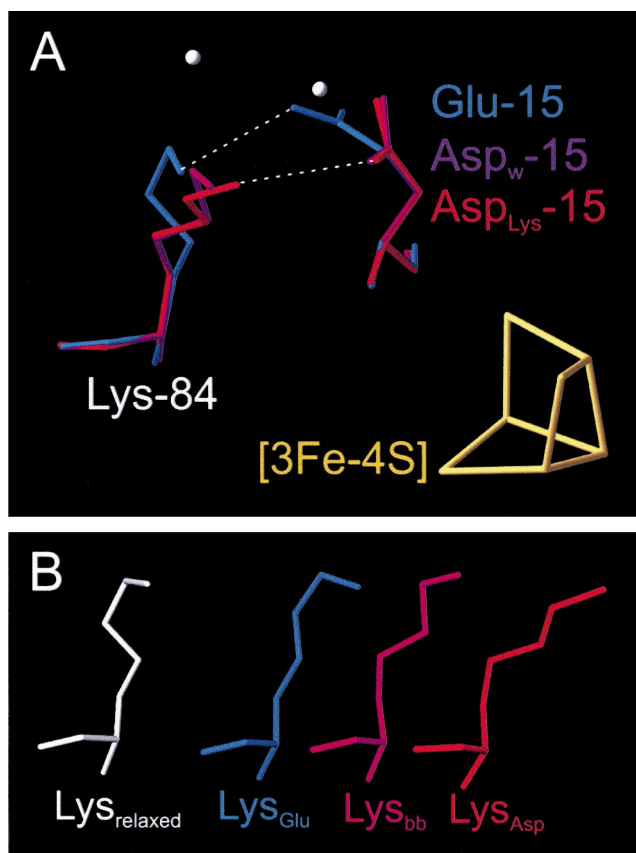


Fig. 1. Conformations of Lys84 and Asp15/Glu15 in *A. vinelandii* ferredoxin I. (A) Mutual arrangement of Lys84 and Asp15/Glu15 in oxidised ferredoxin I. The red structure shows Asp15 that is hydrogen bonded with Lys84 in wild-type ferredoxin I (Asp<sub>Lys</sub>/Lys<sub>asp</sub> state, PDB entry 7FD1, see [2]). The violet structure shows the alternative sub-state where Asp15 and Lys84 do not interact with each other (Asp<sub>w</sub>/Lys<sub>bb</sub> state, PDB entry 7FD1, see [2]). The blue structure shows Lys84 bound to Glu15 in the Asp15→Glu mutant (PDB entry 1D3W, see [1]). The position and structure of the [3Fe-4S] cluster (yellow) are unchanged in all three structures [1,2]. Two water molecules on a HB distance from Asp15 and Lys84, respectively, are depicted in white. The HBs are shown by dashed lines. (B) Comparison of four different conformers of Lys84 in oxidised ferredoxin I. Lys84 is most tensed when it forms a HB with Asp15 (red Lys<sub>asp</sub> conformer, PDB entry 7FD1). In the alternative conformation (violet Lys<sub>bb</sub> conformer, PDB entry 7FD1) Lys84 is partially relaxed. The most relaxed is the conformation in the Asp15→Glu mutant (blue Lys<sub>Glu</sub> conformer, PDB entry 1D3W). The latter conformation is very close to the lowest energy conformation of Lys in water (white Lys<sub>relaxed</sub> conformer, was obtained by MD simulations as described in the text).

frequency of  $10^{10} \text{ s}^{-1}$  [1]. Respectively, the ‘rapid penetrative excursions’ of the Asp15 side chain were suggested as the mechanism of proton transfer in this system [1]. We were intrigued by the finding that the enzyme turnover was blocked even when Asp15 was replaced by a glutamic acid residue (Glu) [1]. Generally, Glu residues are known to be able to serve as mobile proton carriers in various proton pumps [3,4] and to substitute for Asp residues carrying this function [5]. A glutamate introduced at the 15th position (Glu15), with its longer and more flexible side chain, should apparently do even better as a mobile proton carrier in the case of ferredoxin I than Asp15. Here we tackled this puzzling incompetence of Glu15 by molecular dynamics (MD) simulations. As documented below, we revealed that the conformational alterations of Asp15 are energetically balanced by the straining of the nearby Lys84 side chain in wild-type ferredoxin I but not in the Asp15→Glu mutant.

Two almost isoenergetic states have been revealed by X-ray crystallography at 100 K for oxidised wild-type *A. vinelandii* ferredoxin I [1,2]. They differ only in the conformations of the surface Asp15 and Lys84 side chains. Asp15 either forms a hydrogen bond (HB) with the nearby Lys84 (red Asp<sub>Lys</sub>/Lys<sub>asp</sub> conformation in Fig. 1A) or is ‘free’ interacting only with water (violet Asp<sub>w</sub>/Lys<sub>bb</sub> conformation in Fig. 1A). In the latter case Lys84 forms alternative HBs with the backbone carbonyl oxygen atoms of the neighbouring Lys85 and Tyr13 [1,2]. By contrast, similar cryocrystallographic data for the Asp15→Glu mutant revealed a single conformation with a HB between Glu15 and Lys84 [1] (see the blue structure in Fig. 1A).

Because the ‘penetrating excursions’ of the protonated Asp15 towards [3Fe-4S] seem to have a frequency of  $10^{10} \text{ s}^{-1}$  [1], they can hardly limit the wild-type ferredoxin I turnover at approx.  $10^3 \text{ s}^{-1}$ . The latter is rather determined by the intrinsic rate constant of proton exchange at the protein-water interface ( $10^3$ – $10^4 \text{ s}^{-1}$  at neutral pH [6]) and by the probability to find the system in the Asp<sub>w</sub>/Lys<sub>bb</sub> state where the ionic bridge with Lys84 does not prevent the protonation of the carboxyl<sup>1</sup>. The ratio of 2:1 between the populations of Asp<sub>w</sub>/Lys<sub>bb</sub> and Asp<sub>Lys</sub>/Lys<sub>asp</sub> states in the crystals of oxidised ferredoxin I at 100 K [1] reflects a slight shift of the equilibrium

towards the Asp<sub>w</sub>/Lys<sub>bb</sub> state and corresponds to an energy difference of only 2 kJ/mol. This energy poise implies that the energy gain of the HB between Asp15 and Lys84 (a value of 20 kJ/mol could be taken as a reasonable energy estimate for such a salt bridge) is somehow counterbalanced in the Asp<sub>lys</sub>/Lys<sub>asp</sub> conformation. It seemed plausible that such a counterbalance is provided by the stretching of the Lys84 side chain towards Asp15 as seen in Fig. 1A,B (especially in the view of the absence of any other structural differences between two apparently isoenergetic sub-states of the oxidised enzyme [1,2]). Such a stretching of Lys84 is likely to be unfavourable in the polar environment of the protein-water interface.

We verified the latter suggestion by performing MD simulations both of wild-type ferredoxin I and of the Asp15→Glu mutant. For modelling, we took the Asp<sub>lys</sub>/Lys<sub>asp</sub> sub-conformation of wild-type ferredoxin I (7FD1 PDB entry [1,2]) and the structure of the Asp15→Glu mutant (1D3W PDB entry [1]). Each structure was immersed in a water sphere with a diameter of 4.8 nm that contained 1472 water molecules. A spherical harmonic boundary potential was applied to prevent water evaporation. The MD simulation was carried out by the programme NAMD2 [7], using the all-atom empirical force field CHARMM22 [8]. Each system was let to equilibrate for 300 ps at 300 K. For analysis we took the following 1 ns MD simulation tracks.

Fig. 2A shows the time-dependent changes in the distance  $q$  between the NZ nitrogen of Lys84 and the nearest carboxyl oxygen of Asp15 as inferred from MD simulation of wild-type ferredoxin. Fig. 2B shows the analogous trajectory for the Asp15→Glu mutant (here the distance between Lys84 and the

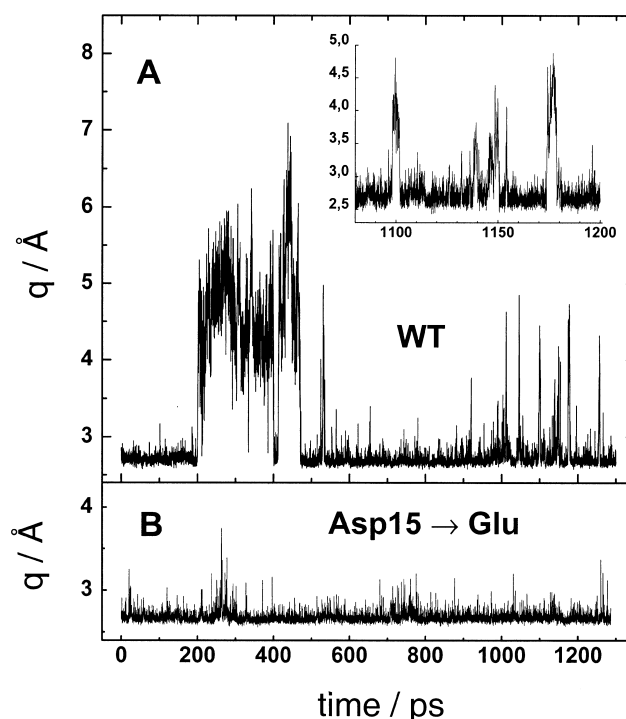


Fig. 2. The time dependence of the distance  $q$  between the NZ nitrogen of Lys84 and the nearest carboxyl oxygen of Asp15 in wild-type ferredoxin I (A), and of the analogous distance between Lys84 and Glu15 in the Asp15→Glu mutant (B) as obtained by MD simulation. The insertion in A shows an extended segment of the MD track (note the different time scales).

nearest carboxyl oxygen of Glu15 is plotted). The dynamic behaviour of the two proteins differed qualitatively. In the Asp15→Glu mutant, the HB between Glu15 and Lys84 was stable throughout the simulation. In wild-type ferredoxin I, the bond broke and restored back many times. Thus, the MD simulation reproduced correctly the existence of two conformational sub-states in the wild-type enzyme but not in the Asp15→Glu mutant (cf. tracks in Fig. 2A,B).

We calculated the distribution functions  $P_{WT}(q)$  and  $P_{Glu}(q)$ . Free energy is known to be connected with the distribution function by the relation  $G(q) = -kT \log P(q) + G_0$  (where  $G_0$  is a constant). The respective calculated energy profiles  $G_{WT}(q)$  and  $G_{Glu}(q)$  are plotted in Fig. 3 by open and closed symbols, respectively (the free energies in the main minima were equalled to zero). In the Asp15→Glu mutant, the energy profile of the Glu15-Lys84 HB

<sup>1</sup> In the mutants where Asp15 was replaced by asparagine, the turnover rate dropped to approx.  $1 \text{ s}^{-1}$  [1]. Such a kinetic behaviour resembles strikingly the proton transfer in the bacterial photosynthetic reaction centre (RC) of the purple phototrophic bacterium *Rhodobacter sphaeroides*. In the RC, the rate constant of the proton transfer from the surface to the reduced secondary quinone acceptor  $Q_B$  decreases from approx.  $10^3$ – $10^4 \text{ s}^{-1}$  in the wild-type to approx.  $1 \text{ s}^{-1}$  in the mutants with the proton transferring residues in the vicinity of  $Q_B$  replaced by non-ionisable ones [12]. It looks as if the non-specific intraprotein proton transfer, presumably via penetrating water molecules, takes generally about 1 s.

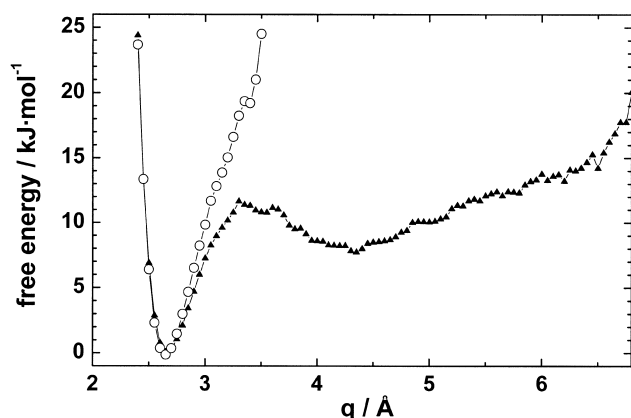


Fig. 3. The free energies of the HBs between Asp15 and Lys84 in wild-type ferredoxin I ( $\Delta$ ) and between Glu15 and Lys84 in the Asp15→Glu mutant ( $\circ$ ) as functions of the coordinate  $q$ . The energy profiles were calculated from the MD data documented in Fig. 2 (see the text for details).

had a single minimum at  $q = 2.64 \text{ \AA}$ . In the wild type, the energy profile of the Asp15-Lys84 HB had two minima at  $q = 2.67 \text{ \AA}$  and  $q = 4.35 \text{ \AA}$ , respectively, with free energies differing by approx.  $7.5 \text{ kJ/mol}$ . A comparison of the profiles at  $q > 3.5 \text{ \AA}$  revealed that the HB in the mutant was at least  $18 \text{ kJ/mol}$  more stable than those in the wild type. The difference between the average distances of HB in the wild type ( $2.70 \text{ \AA}$ ) and in the Asp15→Glu mutant ( $2.67 \text{ \AA}$ ) is equivalent to the effect of stretching force with a magnitude of  $8 \text{ kJ mol}^{-1} \text{ \AA}^{-1}$ .

We have noted that the equilibrium position of the NZ nitrogen of Lys84 in the mutant was shifted by  $1.9 \text{ \AA}$  relative to its equilibrium position in the wild type. Considering this shift as an ‘elastic’ coordinate, we calculated the respective energy profiles using the same procedure which has been applied for the coordinate  $q$  in Fig. 3 (not documented). From these energy profiles, we estimated the free energy needed to bring the NZ nitrogen atom of Lys84 in the Asp15→Glu mutant in the position of its counterpart in the Asp<sub>lys</sub>/Lys<sub>asp</sub> sub-conformation of wild-type ferredoxin I as  $> 20 \text{ kJ/mol}$ . The obtained value agrees with the difference in the HB strength between the Lys84-Asp15 and Lys84-Glu15 ion pairs (cf. above).

We also used another, complementary way to compare the free energies of the different Lys84 conformers. The core of ferredoxin I is very rigid and

does not change either upon reduction or in response to the surface amino acid substitutions [1,2]. As both Asp15 and Lys84 are located on the surface and look into water, they could be considered as being fixed on a water-immersed rigid ‘frame’. It is therefore plausible that the interaction with water may essentially determine the magnitude of free energy difference between the conformers of Lys84. To check this possibility, we ‘cut out’ different Lys84 conformers from respective structures: Lys<sub>Asp</sub> and Lys<sub>bb</sub> were taken from the sub-structures Asp<sub>w</sub>/Lys<sub>bb</sub> and Asp<sub>lys</sub>/Lys<sub>asp</sub> of the 7FD1 PDB entry, whereas the 1D3W PDB entry was used to get the conformation of Lys<sub>Glu</sub>. These conformers, depicted in Fig. 1B, were placed in a water sphere with a diameter of  $16 \text{ \AA}$ . The spherical harmonic boundary potential with a radius of  $7.5 \text{ \AA}$  was applied to prevent water evaporation. The native conformations of Lys84 were fixed during the simulation, whereas water was set free. Because of its small size, the system had a short relaxation time of approx.  $5 \text{ ps}$ . The ultimate thermal equilibration was achieved by  $100 \text{ ps}$  molecular dynamics simulation at  $300 \text{ K}$ ; then the control tracks of  $300 \text{ ps}$  were analysed to calculate the average potential energy of the system (excluding the internal energy of Lys84 itself). The average energy of the system was highest in its Lys<sub>Asp</sub> conformation, namely  $-3224.0 \pm 0.3 \text{ kJ/mol}$ . The energy of the Lys<sub>bb</sub> conformer was  $11 \text{ kJ/mol}$  lower:  $-3235.4 \pm 0.3 \text{ kJ/mol}$ . The energy of the Lys<sub>Glu</sub> conformer was the lowest:  $-3243.0 \pm 0.3 \text{ kJ/mol}$ , i.e.  $19 \text{ kJ/mol}$  lower than that of the Lys<sub>Asp</sub> conformation. We performed analogous calculations with a non-constrained Lys residue (only the backbone atoms were fixed to prevent the ‘escape’ of Lys from the water sphere during the MD simulation). The resulting conformation of a ‘relaxed’ Lys residue was very similar to that of Lys84 in the Asp15→Glu mutant (compare Lys<sub>relaxed</sub> and Lys<sub>Glu</sub> structures in Fig. 1B).

The results of all presented calculations indicate that Lys84 is elastically strained in the Asp<sub>lys</sub>/Lys<sub>Asp</sub> conformation of the ground-state wild-type ferredoxin I. The tension has a magnitude of  $8 \text{ kJ mol}^{-1} \text{ \AA}^{-1}$ , whereas the total elastic energy comprises  $> 18 \text{ kJ mol}^{-1}$ . The unfavourable interaction of the Lys<sub>Asp</sub> side chain with water as compared to the Lys<sub>bb</sub> conformer seems to account for half of this energy. The strain of Lys<sub>Asp</sub> compensates the energy

gain of the HB (salt bridge) between Lys84 and Asp15 and accounts for the comparable energies of the Asp<sub>w</sub>/Lys<sub>bb</sub> and Asp<sub>lys</sub>/Lys<sub>Asp</sub> conformers of oxidised ferredoxin I. This energy poising allows Asp15 to alternate between several isoenergetic conformations and to serve as a mobile proton carrier.

In the case of the Asp15→Glu mutant, the longer side chain of Glu15 reaches Lys84 easily and forms a stable HB. Molecular dynamics simulations revealed that Lys84 is thereby not strained but ‘relaxed’, by contrast with the wild type. The absence of a counterbalance for the strong HB between Glu15 and Lys84 leads to the selective stabilisation of the bonded state by > 18 kJ/mol. As a result, Glu15 is effectively immobilised and cannot serve as a mobile proton carrier. Paradoxically, the *absence* of the strain in the Asp15→Glu mutant seems to disrupt the fragile energy poise which accounts for the enzyme efficiency.

It is noteworthy that the correlation between the structural data and the results of MD simulations, although good, was not absolute. The MD simulation predicted the domination of the Asp<sub>lys</sub>/Lys<sub>asp</sub> sub-conformation in the case of the wild-type enzyme, whereas the X-ray structures (obtained at 100 K) favoured the alternative sub-conformation with the ‘free’ Asp15. The possible reasons for this discrepancy might be either (i) inaccuracy of the atomic force field potentials used on the MD simulation or (ii) a slight shift of the equilibrium between two sub-states on freezing. Furthermore, the MD simulations predicted a shorter HB as compared to the structural data. Because the HB distances between Lys84 and Asp15/Glu15 of approx. 3.0 Å in the X-ray structures [1,2] seem to be unrealistically long for salt bridges, we prefer to consider the HB distances as yielded from MD simulations (approx. 2.7 Å) as more reliable. The latter discrepancy might result from the usage of the library rotamer structures on fitting the X-ray data [1,2].

It is broadly accepted that the energy of substrate binding could be accumulated in the mechanical strain of an enzyme (see [9] and references therein). However, as has been first noted by Vallee and Williams, the strained (entatic) conformations could be peculiar even to the substrate-free, ground-state enzymes [10]. The experimental prove of this concept is limited to the differences between the spectral prop-

erties of metals/metal clusters in various enzymes and their inorganic analogues (see [11] and references therein). As some catalytic side chains showed elevated and specific reactivity towards simple organic reagents unrelated to substrates even in the case of substrate-free, non-redox enzymes (as compared to other similar side chains of the same protein), it has been hypothesised that entatic states could also be involved in these cases (see [11] and references therein)<sup>2</sup>. Lys84 in ferredoxin I of *A. vinelandii* represents, to our knowledge, the first case where the strained (entatic) conformation of a particular amino acid side chain could be directly identified in the ground-state enzyme and assigned to a distinct mechanism of energy balance during the catalytic transition.

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<sup>2</sup> Such an elevated reactivity might be generally coupled with the existence of several isoenergetic conformations of the catalytic site (see [13] for a more detailed discussion of the peculiarity of multiple isoenergetic states to the enzyme catalysts).

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