Structural Basis of Inter-protein Electron Transfer for Nitrite Reduction in Denitrification
Paper in journals : this is the first page of a paper published in ***Nature***.
[*Nature*] 462, 117-120 (2009)

Vol 462|5 November 2009|doi:10.1038/nature08507 nature

LETTERS

Structural basis of inter-protein electron transfer for nitrite reduction in denitrification

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Recent earth science studies have pointed out that massive acceleration of the global nitrogen cycle by anthropogenic addition of bio-available nitrogen has led to a host of environmental problems[1]. Nitrous oxide (N_2O) is a greenhouse gas that is an intermediate during the biological process known as denitrification[2]. Copper-containing nitrite reductase (CuNIR) is a key enzyme in the process; it produces a precursor for N_2O by catalysing the one-electron reduction of nitrite (NO_2^-) to nitric oxide (NO)[3]. The reduction step is performed by an efficient electron-transfer reaction with a redox-partner protein[4–6]. However, details of the mechanism during the electron-transfer reaction are still unknown. Here we show the high-resolution crystal structure of the electron-transfer complex for CuNIR with its cognate cytochrome *c* as the electron donor. The hydrophobic electron-transfer path is formed at the docking interface by desolvation owing to close contact between the two proteins. Structural analysis of the interface highlights an essential role for the loop region with a hydrophobic patch for protein–protein recognition; it also shows how interface construction allows the variation in atomic components to achieve diverse biological electron transfers.

The biological reduction of nitrite ions to gaseous nitrogen monoxide ($NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$) is catalysed by two very different enzymes called haem cd_1-containing nitrite reductase (cd_1NIR) and CuNIR, which are less commonly known by their product gene names, NirS and NirK, respectively[3]. They are mutually exclusive at the species level in the denitrifying bacteria distributed widely on Earth[7]. For the one-electron reduction of NO_2^-, the enzyme is linked to respiratory electron-transfer chains by cytochrome *c* (Cyt *c*) and/or cupredoxin in the cell[8,9]. Although the electrons destined for the enzyme pass through the cytochrome bc_1 complex in the chain, how they pass on from this complex to the enzyme is still a matter of uncertainty. In general, CuNIR folds a homotrimeric structure with two distinct Cu-binding sites through an approximately 37-kilodalton (kDa) monomer unit[10]. The type 1 Cu site (T1Cu) buried within each monomer relays an electron from the redox-partner protein to the catalytic type 2 Cu site (T2Cu), where NO_2^- is reduced to NO. This enzyme is further classified into two subgroups based on the spectroscopic properties of the T1Cu site, which are blue and green[11]. Whereas the physiological redox-partner protein for green CuNIR is pseudoazurin (PAz), the blue CuNIR has been suggested to accept an electron from azurin (Az) or Cyt *c*[11–13].

In a ubiquitous Gram-negative non-fermenting rod, *Achromobacter xylosoxidans* GIFU 1051 (the organism formerly known as *Alcaligenes xylosoxidans*)[14], it has been found that two structural genes (*nirK* and *nirM*) of the blue CuNIR (AxgNIR) and Cyt c_{551} constitute an operon presumed to be a redox-partner pair (Supplementary Fig. 1). The *nirM* gene is also part of the operon that harbours the *nirS* gene coding for cd_1NIR in other denitrifying bacteria, and the product Cyt c_{551} protein acts as a physiological redox partner for cd_1NIR[15]. Recent gene disruption studies on the *nirK* gene-harbouring bacterium *Bradyrhizobium japonicum* have shown that Cyt c_{550} is required for growth under denitrifying conditions[16]. Moreover, earlier work by Zumft and colleagues has demonstrated that CuNIR can function instead of cd_1NIR; this is most likely caused by the use of Cyt *c* as a redox partner in *Pseudomonas stutzeri*[17]. Thus, it is reasonable to assume that not only A2 but also Cyt c_{551} plays a role as an *in vivo* redox partner for AxgNIR.

To elucidate the physiological relationship between AxgNIR and Cyt c_{551}, the formation of a functional electron-transfer complex between these proteins was analysed by stopped-flow kinetics and a crystallographic approach (Fig. 1). The kinetics of electron transfer from the reduced Cyt c_{551} to AxgNIR was monitored at a wavelength of 420 nm originating from a Soret band of the haem *c* group under anaerobic conditions. The rapid decay of the absorbance is due to the oxidation of Cyt c_{551} and concomitant reduction of AxgNIR (Fig. 1a). The second-order electron-transfer rate constant between the two proteins, $(4.8 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$ (s.d.) (Fig. 1a, inset), strongly supports the idea that Cyt c_{551} interacts functionally with AxgNIR as an electron donor. To understand the detailed mechanism further, the crystal structure of the binary complex of AxgNIR with Cyt c_{551} was determined at a resolution of 1.7 ångströms (Å). A model of the complex, which consists of one Cyt c_{551} and one trimeric AxgNIR molecule within an asymmetric unit, contains 8,242 protein atoms, one haem *c* group, and six copper atoms (Fig. 1b; see also Supplementary Figs 2 and 3a). The AxgNIR molecules contribute to major contacts in the crystal lattice and provide only one space for Cyt c_{551}; this is bound to its docking site on a subunit (Sub-I) of AxgNIR without steric hindrance (Supplementary Fig. 3b).

Within the docking interface, the change in solvent-accessible surface area upon complex formation is estimated to be $-529\ Å^2$ for Sub-I and $-553\ Å^2$ for Cyt c_{551}; the gap index (the ratio of gap volume to buried interface solvent-accessible surface area) is 3.55 Å. The haem *c* group partly protrudes from the protein interior of the Cyt c_{551} molecule at two parts: the thioether-bonded substituent linked to pyrrole ring C and the propionate groups on pyrrole rings A and D (Fig. 1c). The Cyt c_{551}-docking site near the T1Cu site of Sub-I and the haem *c* group are in close contact at a 3.5-Å distance between the Cε atom of Met 87 in Sub-I and the edge CBC methyl carbon of haem *c* (Fig. 2a). At least ten amino-acid residues of AxgNIR are associated with at least 11 residues and the haem *c* group of Cyt c_{551} at the interface (Supplementary Table 2). There is no salt bridge at the interface, and only three direct hydrogen bonds were observed. In general, direct hydrogen bonds between the docking

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The following is a comment on the published paper shown on the preceding page.

Atomic Description of Inter-protein Electron Transfer for Biological Nitrite Reduction in the Global Nitrogen Cycle

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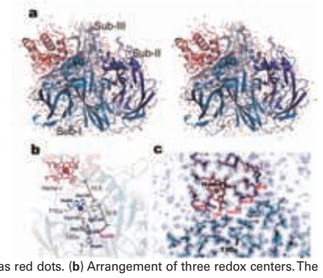

Fig. 1 Overall structure of the interprotein ET complex of AxgNIR with Cyt c_{551}. (**a**) Stereo side view. The Cyt c_{551} molecule is represented as a pink-colored ribbon, and the Cyt c_{551}-docked subunit (Sub-I) of AxgNIR is shown in sky blue, the undocked subunit (Sub-II) in navy, and the other one (Sub-III) in light blue. The heme group (red), T1Cu (dark blue), and T2Cu (gray) are depicted as balls and sticks. Water molecules are shown as red dots. (**b**) Arrangement of three redox centers. The heme group and ligand residues of both Cu centers are shown as sticks. The distances from the CBC methyl group on heme *c* to T1Cu and from T1Cu to T2Cu are given in angstroms. Marks A to D of the heme are indicated the four pyrrole rings. (**c**) Electron density map of the docking interface between AxgNIR (blue) and Cyt c_{551} (pink).

Introduction

Protein electron transfer (ET) reactions play a critical role in biologically vital processes in living cells, most notably respiration and photosynthesis. The reactions occur between protein-bound prosthetic groups separated by long distances, often greater than 10 Å and display high efficiency and specificity. In addition to a dependence on factors inherent to the long-range ET processes, numerous studies have revealed that intermolecular ET reactions requiring a balance of specific binding and fast dissociation are highly sensitive to protein association modes and their protein-protein interfaces. Hence, the conformational changes of the amino acid residues and the behavior of solvent molecules at the interface formed by redox-partner proteins have the potential to regulate inter-protein ET[1].

Recent earth science and geochemical studies have pointed out that the massive acceleration of the global nitrogen cycle as a result of the production and industrial use of artificial nitrogen fertilizers worldwide has led to a host of environmental problems, ranging from eutrophication of terrestrial and aquatic systems to global acidification[2]. Denitrification is one of the biological processes contributing to the maintenance of the nitrogen balance on the earth. Dissimilatory copper-containing nitrite reductase (CuNIR) is a key enzyme in denitrification, catalyzing one-electron reduction of nitrite (NO_2^-) to nitrogen monoxide (NO)[3]. The reaction is specifically regulated by the efficient ET reaction with a redox-partner protein. CuNIRs fold a trimeric structure with two distinct Cu sites per a ca. 37-kDa monomer unit. The type 1 Cu site (T1Cu) buried within each monomer relays an electron from the redox-partner protein to the catalytic type 2 Cu site (T2Cu), where NO_2^- is reduced to NO. Despite much effort by several groups, a crystal structure of the protein–protein complex between CuNIR and its redox-partner has not yet been determined.

Crystal structure of a transient ET complex of CuNIR with Cyt *c*

The crystal structure of a binary complex of CuNIR (AxgNIR) with its redox partner cytochrome c_{551} (Cyt c_{551}) from a ubiquitous gram-negative non-fermenting rod, *Achromobacter xylosoxidans* GIFU1051 (the organism formerly known as *Alcaligenes xylosoxidans*), was determined at a resolution of 1.7 Å. A model of the binary AxgNIR:Cyt c_{551} complex, which consists of one Cyt c_{551} and one AxgNIR molecule within an asymmetric unit, contains 8,242 protein atoms, one heme *c* group, six copper atoms, and 1,073 solvent molecules (Fig.1). Only one Cyt c_{551} molecule is bound to one subunit (Sub-I) of trimeric AxgNIR. The high-resolution crystal structure allows an accurate description of the complex interface between Sub-I and Cyt c_{551}. Within the interface, the change in solvent-accessible surface area upon complex formation is estimated to be -529 Å^2 for Sub-I and -553 Å^2 for Cyt c_{551}. In the isolated Cyt c_{551} structure, the heme c group is partially exposed to solvent at the two parts: the thioether-bonded substituent linked to pyrrole ring C and the propionate groups on pyrrole rings A and D (Fig.1b). At the center of the interface, the Cyt c_{551} docking site near the T1Cu site of Sub-I and the heme *c* group are in close contact at a 3.5-Å distance between the Cε atom of Met87 in Sub-I and the edge CBC methyl carbon of the thioether-bonded substituent on heme *c*. At least 10 amino acid residues of AxgNIR are associated with at least 11 amino acid residues and the heme *c* group of Cyt c_{551} at the interface. The atoms of these residues and heme *c* interact with each other at a distance of ≤3.5 Å. There is no salt bridge at the interface, and only three direct hydrogen bonds were observed: Thr192 Oγ1/Gly67 O (2.8 Å), Glu195 Oε1/Ala69 N (2.8 Å), and Gly198 N/Ser18 O (2.9 Å) of Sub-I/Cyt c_{551}. In general, direct hydrogen bonds between the docking proteins seem to be unfavorable for a transient ET complex because of energetically disadvantageous desolvation[4]; namely, interface of the reaction center (RC):Cyt c_2 complex also has three intermolecular hydrogen bonds[5] and that of the yeast cytochrome *c* peroxidase (CcP):Cyt c complex one hydrogen bond[6], but that of the cytochrome bc_1 (Cyt bc_1):Cyt *c* complex has no hydrogen bond[7].

Interface between CuNIR and Cyt *c*

The building blocks of the interface between AxgNIR and Cyt c_{551} are shown in Fig. 2. The interacting residues of AxgNIR are localized both at the hydrophobic patch near the T1Cu site and at the "tower loop" region extending toward the T1Cu site. These primarily non-polar and neutral residues indicate that hydrophobic and van der Waals interactions strongly contribute to complex for-

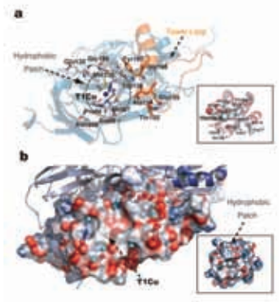

Fig.2 Docking interface of the AxgNIR:Cyt c_{551} complex. (**a**) The building block of the interface. The amino acid residues at the protein interface and the T1Cu ligands are represented as sticks. The T1Cu ligands are colored in blue, the residues involved in the hydrophobic patch in gray, and the residues of the "tower loop" in orange. Inset: the residues around the heme group in Cyt c_{551}. The heme group is shown as spheres. (**b**) Electrostatic potentials of contact protein surfaces. Twenty-five water molecules at the interface are represented as spheres. Eight water molecules bridging between the partner proteins through hydrogen bonds are colored in cyan and the other waters binding to Sub-I or Cyt c_{551} in red.

mation. Contact between these hydrophobic patches brings the redox centers of heme *c* and T1Cu within 10.5 Å, which are close enough to allow for rapid ET[8,9]. Furthermore, 25 water molecules are located at the docking interface. Eight waters bridging the two proteins through hydrogen bonds stabilize the partner proteins, and the remaining waters also provide stabilization through hydrogen bonds and van der Waals contacts to either Sub-I or Cyt c_{551} (Fig.2). All of the water molecules form a characteristic semi-circle around the hydrophobic patch, and the non-polar core interface is sealed off from the aqueous environment.

Superposition of the structure of Cyt c_{551}-docked Sub-I onto that of undocked Sub-II or Sub-III shows an average root-mean-square deviation (rmsd) of 0.7 Å between the corresponding side chain atoms of all the amino acid residues. The most significant structural changes at the interface occur in residues Met87, Met135, Glu195, and Tyr197 (Fig.3). The side chains of Met135 in Sub-II and Sub-III are directed toward solvent. Moreover, HOH516-B in Sub-II and HOH785-C in Sub-III hydrogen bond to the Nε2 atoms of the corresponding solvent-exposed His139 ligands of the T1Cu site within the range of a typical NH-O bond distance (ca. 2.8 Å). On the other hand, Cyt c_{551}-docked Sub-I has the Met135 side chain tilted toward the imidazole ring of the His139 ligand (Fig.3). The Sδ atom of Met135 is within 3.2 Å of the Nε2 atom of the His139 ligand, and consequently the His139 ligand is no longer exposed to solvent. This behavior of Met135 induced by Cyt c_{551} docking brings about van der Waals interactions between the side chain of Met135 and the non-polar side chains of Val22 and Val28 at the hydrophobic patch around the heme-edge CBC (distance between Cγ2 of Val22 and Sδ of Met135, 3.9 Å; distance between Cγ1 of Val28 and Sδ of Met135, 4.1 Å). In Sub-I, Met135 is therefore sandwiched between the Val residues and tightly fixed over the imidazole group of the His139 ligand; that is, the water molecule bound to the Nε2 atom of the histidyl imidazole ring is removed by movement of the side chain of Met135 and its sulfur atom is in a van der Waals contact with the Nε2 atom. These findings support the locations of three Met residues in the NMR structure the CuNIR (AfNIR):pseudoazurin (PAz) complex from *Alcaligenes faecalis*[10]. In the complex, instead of the two Val residues in the AxgNIR:Cyt c_{551} complex, Met16 and Met84 of PAz closely contact to the Met141 residue corresponding to Met135 in AxgNIR. Therefore, in AfNIR:PAz complex, the Met141 tilts toward the His ligand and fixes it. In general, the redox potential of T1Cu is considerably changed by perturbations of the solvent-exposed His ligand such as protonation and π-π interactions[11]. The redox potentials of the T1Cu site of AxgNIR and the heme *c* group of Cyt c_{551} have been independently determined to be +241 and +290 mV vs. NHE at pH 6.0, respectively. Accordingly, it is likely that the docking of Cyt c_{551} onto AxgNIR tunes the redox potential of T1Cu through structural constraint and desolvation of the His139 ligand, allowing a smooth interprotein ET reaction. The similar redox potential tuning has been proposed for the T1Cu sites of amicyanins (Am's) in the binary methylamine dehydrogenase (MADH):Am complex and the ternary MADH:Am:Cyt c_{551i} complex[12].

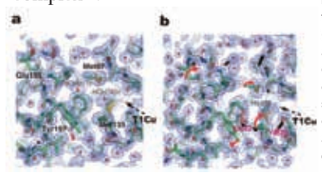

Fig.3 Conformational differences between the Cyt c_{551}-docked and undocked subunits. (**a**) The Cyt c_{551}-docked Sub-I represented as gray sticks is superposed on the undocked subunit (Sub-III) represented as green sticks. (**b**) Sub-III depicted as green sticks is superposed on Sub-I depicted as gray sticks. The conformational changes of amino acid side chains in Sub-I having the contact with Cyt c_{551} are indicated with red arrows. Val22 and Val28 close to Met135 in Cyt c_{551} are shown as pink sticks.

Dominant ET pathway

PATHWAY analysis[13,14] of the AxgNIR:Cyt c_{551} complex was performed to determine the most efficient predicted ET pathway from heme *c* to T1Cu. For the purposes of this analysis, the ET donor was defined as the entire heme *c* group so that the pathway would begin at the most advantageous position on heme *c*. The predicted pathway through the entry/exit port inside the hydrophobic patches of the interfaces is exhibited in Fig. 4. An electron that leaves iron via the exposed CBC methyl group in the AxgNIR:Cyt c_{551} complex is directly transferred to the Cδ atom of Pro88 of AxgNIR by a through-space jump and then shifts from Pro88 to T1Cu through the His89 ligand. The ET pathway represents the most favorable route between the redox centers in the core of the hydrophobic interface.

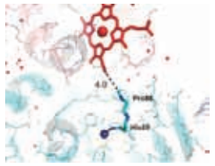

Fig.4 Theoretically dominant ET pathway between heme *c* and T1Cu. Interprotein ET pathway in the AxgNIR:Cyt c_{551} complex. The best pathway is shown as a broken-line (through-space process) and sticks (through-bond process). The distance of through-space jump between the CBC methyl group and the Cδ atom of Pro88 is given in angstrom. The heme group (red), the T1Cu atom (dark blue), and waters (red) are depicted as balls and sticks.

Conclusion

Recognition and interaction between the protein surfaces, as observed in the transient donor-acceptor (Cyt c_{551}-AxgNIR) complex structure occur through sufficient specificity of polar and non-polar interactions, providing a minimal site at the core of the protein–protein interface that ensures the geometry suited for ET reaction. It is particularly important for a deeper understanding of biological ET processes to explore how interface constructions for efficient ET reaction vary with protein–protein shape complementarity, surface charge and polarity, and dynamic fluctuations of the proteins and the organized water molecules at the interface.

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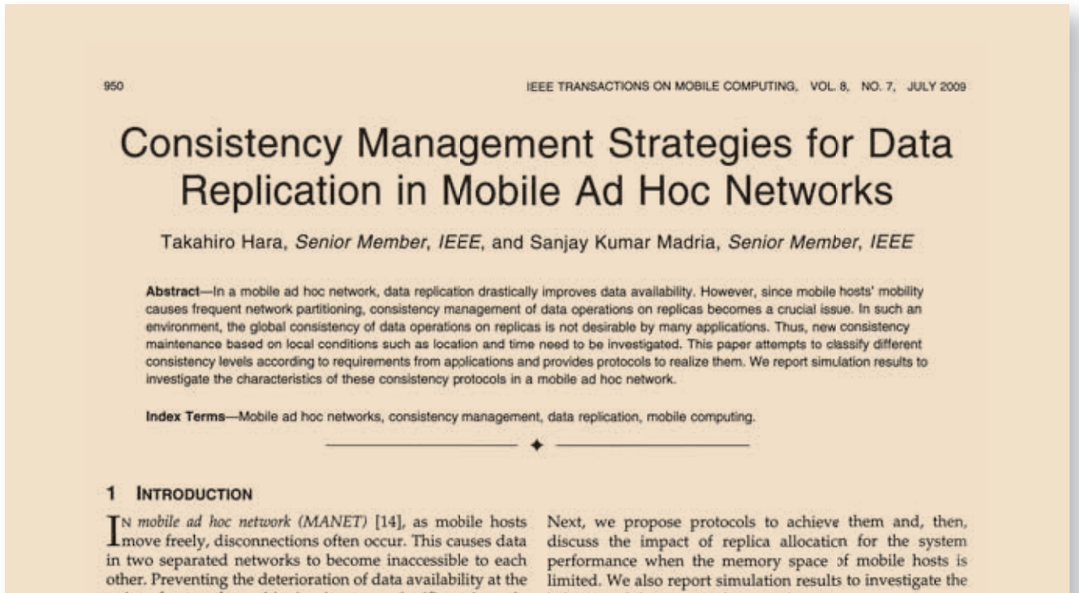
Consistency Management Strategies for Data Replication in Mobile Ad Hoc Networks
Paper in journals : this is the first page of a paper published in ***IEEE Transactions on Mobile Computing.***
[***IEEE Transactions on Mobile Computing***] 8(7), 950-967 (2009)



950 IEEE TRANSACTIONS ON MOBILE COMPUTING, VOL. 8, NO. 7, JULY 2009

Consistency Management Strategies for Data Replication in Mobile Ad Hoc Networks

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Abstract—In a mobile ad hoc network, data replication drastically improves data availability. However, since mobile hosts' mobility causes frequent network partitioning, consistency management of data operations on replicas becomes a crucial issue. In such an environment, the global consistency of data operations on replicas is not desirable by many applications. Thus, new consistency maintenance based on local conditions such as location and time need to be investigated. This paper attempts to classify different consistency levels according to requirements from applications and provides protocols to realize them. We report simulation results to investigate the characteristics of these consistency protocols in a mobile ad hoc network.

Index Terms—Mobile ad hoc networks, consistency management, data replication, mobile computing.

1 Introduction

In *mobile ad hoc network (MANET)* [14], as mobile hosts move freely, disconnections often occur. This causes data in two separated networks to become inaccessible to each other. Preventing the deterioration of data availability at the point of network partitioning is a very significant issue in MANETs [9], [15]. To improve data availability, data replication is the most promising solution [4], [8]. Based on this idea, we have designed effective data replication techniques in MANETs in our previous papers [9], [10], [12].

In [10] and [12], we assume that replicas of a data item become invalid after the host holding the original updates it and the consistency of data operations on replicas is kept in the entire network. However, since network partitioning frequently occurs in a MANET, this strong consistency management scheme heavily deteriorates the data availability. Moreover, many applications in MANETs do not require such a strong consistency. For instance, consider a situation where members of a rescue service that constructs a MANET in the disaster area are divided into several groups each of which is responsible of a certain region and the members in each group share various kinds of information such as that on the extent of damages. In this situation, the consistency of data operations to data items that are used locally in each group must be strictly kept in the same group and is not required to be maintained strictly in different groups.

In this paper, we discuss different consistency conditions of data operations on replicas in MANETs. First, we classify consistency levels according to application requirements. Next, we propose protocols to achieve them and, then, discuss the impact of replica allocation for the system performance when the memory space of mobile hosts is limited. We also report simulation results to investigate the behavior of the proposed protocols.

It should be noted that our proposed consistency levels and protocols for achieving them are not very novel because these are basically common and simple approaches to maintain the consistency based on a typical quorum system and time-based coherency condition. The main contributions of this paper are not only the proposal of the consistency levels and protocols but 1) the classification of consistency levels according to the system and application requirements, 2) the choices of the existing techniques and their extensions for design of the protocols of these consistency levels in MANETs, and 3) performance studies of these protocols.

Note that some of the results of this paper have been reported in [11].

2 Related Work

Consistency management is a popular research topic in distributed database systems. For example, Alonso et al. [1] discussed cache coherency issues and classified several coherency conditions such as time-based, value-based, and version-based ones. As mentioned in Section 1, our proposed consistency levels are basically based on conventional approaches. Time-based Consistency (TC) in this paper is similar to the time-based coherency condition, *default coherency condition*, in [1]. There have been also many conventional works that aim to weaken the consistency such as *Epsilon serializability*, which is a generalization of classic serializability and allows some limited amount of inconsistency [21]. Some of these conventional approaches are applicable to our proposed consistency levels, which is open to our future work.

In mobile environments (not MANET), several consistency management strategies that consicer host disconnections have been proposed [13], [17], [19], [20]. Most of them

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Manuscript received 25 Dec. 2006; revised 24 Oct. 2007; accepted 12 Apr. 2008; published online 15 Oct. 2008.
For information on obtaining reprints of this article, please send e-mail to: tmc@computer.org, and reference IEEECS Log Number TMC-0344-1206.
Digital Object Identifier no. 10.1109/TMC.2008.150.

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