Truncated hemoglobins (trHbs) are small hemoproteins forming a separate cluster within the hemoglobin superfamily; their functional roles in bacteria, plants, and unicellular eukaryotes are marginally understood. Crystalllographic investigations have shown that the trHb fold (a two-on-two α-helical sandwich related to the globin fold) hosts a protein matrix tunnel system offering a potential path for ligand diffusion to the heme distal site. The tunnel topology is conserved in group I trHbs, although with modulation of its size/structure. Here, we present a crystalllographic investigation on trHbs from *Mycobacterium tuberculosis*, *Chlamydomonas eugametos*, and *Paramecium caudatum*, showing that treatment of trHb crystals under xenon pressure leads to binding of xenon atoms at specific (conserved) sites along the protein matrix tunnel. The crystalllographic results are in keeping with data from molecular dynamics simulations, where a dioxygen molecule is left free to diffuse within the protein matrix. Modulation of xenon binding over four main sites is related to the structural properties of the tunnel system in the three trHbs and may be connected to their functional roles. In a parallel cryo-electron microscopy and cryo-electron tomography investigation on *Mycobacterium tuberculosis* trHbN, we show that butyl isocyanide also binds within the apolar tunnel, in excellent agreement with concepts derived from the xenon binding experiments. These results, together with recent data on atypical CO rebinding kinetics to group I trHbs, underline the potential role of the tunnel system in supporting diffusion, but also accumulation in multiple copies, of low polarity ligands/molecules within group I trHbs.

Truncated hemoglobins (trHbs) are small oxygen-binding hemoproteins, identified in bacteria, higher plants, and in certain unicellular eukaryotes, building a separate cluster within the hemoglobin superfamily. Based on amino acid sequence analysis, three trHb phylogenetic groups (I, II, and III) have been recognized (1). TrHbs display amino acid sequences that are 20–40 residues shorter than those of vertebrate hemoglobins, but they share large sequence similarities within the same group, may share less then 20% amino acid sequence identity (1) (Fig. 1). TrHbs from more than one group can coexist in some bacteria, suggesting a wide diversification of functions. Possible trHb functions that are consistent with observed biophysical properties include long term ligand or substrate storage, NO detoxification, O$_2$/NO sensor, redox reactions, and O$_2$ delivery under hypoxic conditions (1–5). In *Mycobacterium bovis BCG*, trHbN promotes an efficient dioxygenase reaction whereby NO is converted to nitrate by the oxygenated heme (4).

So far, four group I trHbs from *Chlamydomonas eugametos* (Ce-trHb), *Paramecium caudatum* (Pc-trHb), *Mycobacterium tuberculosis* (Mt-trHbO), and *Synechocystis* sp. (Ss-trHb) and one group II trHb from *M. tuberculosis* (Mt-trHbN) have been structurally characterized (5–9). The main structural features highlighted by these studies are an unprecedented two-on-two α-helical sandwich fold, a ligand-dependent hydrogen-bonding network within the distal heme pocket, and a protein matrix hydrophobic tunnel/cavity network connecting the solvent space to the heme distal pocket through one or two access sites (5, 6). Such tunnel/cavity network is topologically conserved in Ce-trHb, Pc-trHb, and in Mt-trHbN three-dimensional structures, being built by almost invariant apolar residues (5, 6) (Fig. 1). In contrast, the recently solved crystal structure of hexacoordinated Ss-trHb, where the sixth heme ligand is residue HisE10, did not show evidence of such cavity network, likely related to extensive conformational changes observed in the protein distal site region (8).

In Mt-trHbN, the tunnel is composed of two orthogonal branches, yielding a L-shaped path through the protein matrix.

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* The abbreviations used are: trHb, truncated hemoglobin; Ce-trHb, group I trHb from *C. eugametos*; Mt-trHbO, group I trHb from *M. tuberculosis*; Mt-trHbO, group II trHb from *M. tuberculosis*; Pc-trHb, group I trHb from *P. caudatum*; Ss-trHb, group I trHb from *Synechocystis* sp.; Mb, myoglobin; MD, molecular dynamics; r.m.s.d., root mean square deviation.

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The tunnel short branch (about 8 Å long) connects the heme distal site to the outer solvent space, at a location comprised between the central region of the G and H helices. The tunnel long branch stretches for about 20 Å through the protein matrix, from the heme distal cavity to a solvent access site located between the interhelical AB and GH loops. Overall, the tunnel volume is about 265 Å³ (6). A very similar but more open tunnel system displays a volume of about 400 Å³ in Ce-trHb, whereas in Pc-trHb the tunnel is restricted to three cavities, with an overall volume of 180 Å³ (5). Small cavities have been observed in sperm whale myoglobin (Mb) and are recognized to act as temporary storage and/or accumulation (1, 6, 9, 15). To shed first light on such stimulating ideas, we have analyzed crystals of Mt-trHbN, Ce-trHb, and Pc-trHb treated with xenon or butyl isocyanide, two distinct probes expected to diffuse differently through the trHb tunnel system, and determined their three-dimensional structures. Our data, complemented by molecular dynamics (MD) simulations, show that diffusion to the heme distal site in the hydrophobic tunnel system, where xenon and butyl isocyanide simulations, suggest important roles in controlling diffusion of small ligands to/from the heme distal pocket or in temporary ligand storage and/or accumulation (1, 6, 9, 15). To shed first light on such stimulating ideas, we have analyzed crystals of Mt-trHbN, Ce-trHb, and Pc-trHb treated with xenon or butyl isocyanide, two distinct probes expected to diffuse differently through the trHb tunnel system, and determined their three-dimensional structures. Our data, complemented by molecular dynamics (MD) simulations, show that diffusion to the heme distal site in the group I trHbs considered may follow a path matching the trHb hydrophobic tunnel system, where xenon and butyl isocyanide docking sites are experimentally identified.

EXPERIMENTAL PROCEDURES

Mt-trHbN, Ce-trHb, and Pc-trHb were expressed, purified, and crystallized as previously reported (5, 6). To promote xenon diffusion within the protein matrix tunnel, the selected trHb crystals, in their cryo-protectant system, the cryo-protectant solution supplemented with 20% (v/v) glycerol, as cryo-protectant. The diffraction data (to 2.1 Å resolution) were processed and scaled using MOSFLM (17) and SCALA (18). Structure solution and refinement were carried over as described above for the xenon adduct; however, to minimize local bias, an Ala-trimmed model of Mt-trHbN was used as the starting model. A prominent difference electron density peak next to the A subunit heme distal cavity suggested the presence of butyl isocyanide and was refined accordingly (for the final model, R factor = 19.4% and Rfree = 26.8%, at 2.45 Å resolution). Monomeric Ce-trHb-refined (R factor = 12.6% and Rfree = 28.2%, at 2.40 Å resolution) xenon adducts display 4 and 1 protein matrix xenon atoms, respectively.

Mt-trHbN cyanomethyl crystals were soaked for about 30 min in a solution containing the stabilizing medium (1.46 M K2HPO4, 0.40 M KH2PO4, 0.001 M KCN, pH 8.1) and 0.038 M butyl isocyanide. X-ray diffraction data were collected at 100 K, at European Synchrotron Radiation Facility beam line ID14-2 (Grenoble, France), using the soaking solution supplemented with 20% (v/v) glycerol, as cryo-protectant. The diffraction data (to 2.1 Å resolution) were processed and scaled using MOSFLM (17) and SCALA (18). Structure solution and refinement were carried over as described above for the xenon adduct; however, to minimize local bias, an Ala-trimmed model of Mt-trHbN was used as the starting model. A prominent difference electron density peak next to the A subunit heme distal cavity suggested the presence of butyl isocyanide and was refined accordingly (for the final model, R factor = 19.4% and Rfree = 26.8%, at 2.45 Å resolution). Monomeric Ce-trHb-refined (R factor = 12.6% and Rfree = 28.2%, at 2.40 Å resolution) xenon adducts display 4 and 1 protein matrix xenon atoms, respectively.

Similar experiments were performed on Ce-trHb and on Pc-trHb, soaking the crystals in stabilizing solutions containing 0.020 M butyl isocyanide, for 2 h. In the case of Ce-trHb, a 3.0 Å resolution data set was collected in house. Inspection of the difference Fourier maps showed an elongated difference electron density peak in the long branch of the protein matrix tunnel, allowing only a qualitative modeling of a bound butyl isocyanide molecule, because of low occupancy (data not shown). In the case of the butyl isocyanide Pc-trHb-soaked crystals (data collected in house, up to 2.4 Å resolution), no extra electron density could be located, indicating the lack of butyl isocyanide binding to this trHb. Table I summarizes all the data collection and refinement statistics. The atomic coordinates and structure factors for the above trHb adducts have been deposited with the Protein Data Bank with the entry codes 1a56 (Mt-trHbN xenon), 1uux (Ce-trHb xenon), 1ivy (Pc-trHb xenon), and 1a61 (Mt-trHbN butyl isocyanide).

The MD simulations of dioxygen diffusion within the Mt-trHbN...
Ligand Tunneling in Truncated Hemoglobins

**Table I**

Data collection and refinement statistics

|                     | Mt-trHbN-butyl ESRF ID14-2 | Mt-trHbN-Xe CuKα | Ce-trHb-Xe CuKα | Pe-trHb-Xe CuKα |
|---------------------|----------------------------|-------------------|-----------------|-----------------|
| Wavelength (Å)      | 0.933                      | 1.542             | 1.542           | 1.542           |
| Resolution (Å)      | 20–2.10                    | 40–2.43           | 30–2.45         | 20–2.40         |
| Unique reflections  | 13,707                     | 9,345             | 4,801           | 4,985           |
| Multiplicity        | 3.9 (3.7)†                 | 4.3 (4.0)‡        | 3.6 (3.3)†      | 2.1 (1.9)‡      |
| Completeness (%)    | 92.2 (92.2)                | 94.9 (94.9)       | 98.4 (95.4)     | 98.3 (98.7)     |
| Mosaicity (°)       | 1.3                        | 0.9               | 0.98            | 1.08            |
| Rsym (%)            | 10.9 (35.1)                | 9.8 (27.7)        | 15.4 (43.2)     | 11.2 (36.0)     |
| Space group         | P212121                    | P212121           | P4               |                 |
| a (Å)               | 43.5, b = 61.4, c = 91.8   | 44.3, b = 62.0, a = 34.5, b = 52.9, a = 61.4, c = 33.8 |
| Refinement          |                            |                   |                 |                 |
| R factor/Rw (%)     | 19.4/25.1†                 | 19.4/27.7‡        | 21.0/26.8‡      | 21.1/25.2‡      |
| r.m.s.d. (Å)        | 0.012                      | 0.009             | 0.003           | 0.011           |
| Angles (°)          | 1.335                      | 1.224             | 1.9             | 6.9             |
| Ramachandran plot†  | 98.6                       | 96.4              | 98.1            | 93.1            |
| Most favored (%)    | 1.4                        | 3.6               | 1.9             |                 |
| Additionally allowed (%) |                 |                   |                 |                 |
| B factors (Å²)      | 29                         | 32                | 31              | 47              |
| Protein             | 34                         | 27                | 26              | 45              |
| Solvent             | 43 [1.0]                   | 44 [0.7]          | 37 [0.8]        |                 |
| Xe1                 | 43 [0.6]                   | 35 [0.8]          |                 |                 |
| Xe2                 | 31 [0.5]                   | 31 [0.5]          |                 |                 |
| Xe4                 | 44 [0.5]                   | 58 [0.4]          |                 |                 |
| Xe6                 | 61 [0.3]                   | 59 [0.7]          |                 |                 |
| Butyl isocyn        | 51 [1.0]                   |                   |                 |                 |

† Outer shell statistics Mt-trHbN-butyl isocyn (2.11–2.10 Å) within parentheses.
‡ Outer shell statistics Mt-trHbN-Xe (2.56–2.43 Å) within parentheses.
§ Outer shell statistics Ce-trHb-Xe (2.49–2.45 Å) within parentheses.
¶ Outer shell statistics Pe-trHb-Xe (2.44–2.40 Å) within parentheses.
Ο Calculated using 5% of the reflections.
Φ Calculated using 10% of the reflections.
* Generated using the program PROCHECK (32).

RESULTS

**TrHb Xenon Derivatives**

Mt-trHbN—Analysis of the Mt-trHbN xenon adduct allowed identification of 8 xenon atoms, with different occupancy levels, distributed between the two asymmetric unit molecules. The overall Mt-trHbN structure is little affected by xenon binding, the r.m.s.d. values calculated for comparisons of the xenon bound/unbound A and B subunits being 0.82 and 0.68 Å (on 122 Ca atom pairs), respectively. Three major xenon sites (named Xe1, Xe2, and Xe4; Fig. 2A) are equally present and located in both Mt-trHbN A and B subunits. The Xe1 site (100% xenon occupancy) is located in the tunnel long branch, at 13.3 Å from the heme iron atom, whereas site Xe2 (60% occupancy) falls in the short branch (at 6.3 Å from the heme iron atom). The Xe4 site (50% occupancy) is located at the tunnel short branch entrance, in a hydrophobic cleft between symmetry related molecules. In contrast, the Xe3 site (50% occupancy) is present in subunit B only, being located between the Xe2 and Xe4 sites, at the interface between the tunnel short branch and the solvent. Xe5, located at 9.0 Å from the heme iron atom, between the sites Xe1 and Xe2 in subunit A, displays only 30% occupancy, likely representing a transition site bridging between the Xe1 and Xe2 sites. Such a bridging role is also suggested by the elongated distribution of the residual F – Fc map, around Xe5.

The Xe2, Xe3, and Xe4 sites outline the path of the Mt-trHbN tunnel short branch from the heme distal cavity to the solvent (Fig. 2A) (6). In particular, the Xe3 site protein surface location suggests that access to the heme distal site may occur through an entry site comprised between the G and H helices, defined by residues PheG5(91), AlaG9(95), LeuH8(116), IleH11(119), and AlaH12(120). Binding of xenon at the Xe3 site induces shifts of 0.4–1.2 Å at residues AlaG9(95) and IleH11(119), resulting in a wider solvent aperture for the tunnel short branch. The structural analysis also shows that the Mt-trHbN tunnel long branch is accessible from the solvent, in both A and B subunits, through an aperture defined by residues IleA15(19), IleB2(25), ValB5(28), and LeuG16(102). The location of a full occupancy xenon atom in the Xe1 cavity, at 5.5 Å from the long branch solvent aperture, is stabilized by van der Waals’ contacts with residues ValB5(28), PheE15(62), LeuE19(66), LeuG12(98), and LeuG16(102) (Figs. 1 and 2A).

Ce-trHb—The Ce-trHb structure displays the wider (~5 Å in diameter) and more extended L-shaped tunnel observed so far in group I trHbs (Fig. 2B). The four xenon atoms found in the Ce-trHb xenon adduct follow the long branch tunnel, at mutual distances of 4–5 Å. In particular, three xenon atoms match the Xe1, Xe2, and Xe5 sites observed in Mt-trHbN (the Mt-trHbN-Xe site numbering scheme is kept for Ce-trHb and PctrHb, to identify topologically equivalent xenon sites within the trHb fold). The Xe1 site (70% occupancy) falls at 13.7 Å from the heme iron atom, contacting residues LeuE15(49), LeuE19(53), and LeuG16(91). The Xe2 site (occupancy of 80%) falls at 6.8 Å from the iron atom, being surrounded by residues...
Structural comparison of the Ce-trHb in the presence and in the absence of xenon atoms does not highlight significant main readjustments in the protein backbone (r.m.s.d. of 0.18 Å, calculated over 121 Ca pairs). However, xenon binding induces a 1.0 Å shift of LeuG12(87), such that the tunnel volume is increased from 320 Å³ in the absence of Xe to 380 Å³ in the presence of Xe, originating the Xe5 site. In contrast, the Xe1, Xe2, and Xe6 atoms are found in cavities that are already present in the Xe-free Ce-trHb structure (5). The tunnel short branch of Mt-trHbN is more properly defined as a cavity in Ce-trHb (about 90 Å³), lined by residues PheG5(80), GlnG6(81), AlaG9(84), MetH8(105), ValH11(108), and AlaH12(109). Remarkably, upon xenon binding, such a cavity shrinks to about 50 Å³, because of the conformational readjustments at LeuG12(87) and MetH8(105), linked to occupation of the Xe5 site.

**Pc-trHb**—The residue selection at the B5 and H7 topological sites (Fig. 1), together with a shift of the B6–B10 polypeptide segment, restrict the tunnel size in Pc-trHb and divide it into three cavities (overall volume 180 Å³), topologically distributed along the tunnel long branch described above (Fig. 2C). In agreement with such picture, only one xenon atom is bound by Pc-trHb, at the Xe1 site (80% occupancy, at 13.8 Å from the heme iron atom), in a 65 Å³ cavity lined by the hydrophobic residues ValB2(12), ValB5(15), ThrB6(16), LeuE15(49), LeuE19(53), LeuG12(85), LeuG16(89), and ThrH7(102). As for Mt-trHbN and Ce-trHb, the r.m.s.d. value, calculated for the protein backbones of the aquo-met Pc-trHb and its xenon adduct (0.28 Å, for 116 Ca pairs), is indicative of minor overall structural readjustments. Binding of the xenon atom, however, results in a wider Xe1 cavity (volume increase of 10 Å³), reflected by side chain shifts (of about 0.5 Å) of residues LeuE15(49) and LeuG12(85). In Pc-trHb, these residues build a narrow neck between the Xe1 site and the adjacent (smaller) cavity, corresponding to the Xe2 site in Ce-trHb. The small size of the potential Xe2 site in Pc-trHb (11 Å³) prevents xenon binding. However, xenon binding to Pc-trHb shifts the heme vinyl CBC atom by 0.7 Å to form an additional small cavity with a volume of 12 Å³. This additional cavity is not observed in the aquo-met Pc-trHb structure in the absence of Xe.

**Mt-trHbN-butyl Isocyanide Derivative**

Under the experimental conditions applied, binding of butyl isocyanide to Mt-trHbN occurs essentially only in the tunnel long branch of the A subunit (with 100% occupancy). The B subunit displays electron density compatible with very low occupancy binding only. The r.m.s.d. calculated between subunits A and B of the Mt-trHbN adduct is 0.93 Å, (over Ca pairs 3–125). Butyl isocyanide is completely buried within the short tunnel branch and the heme distal cavity (Figs. 1 and 2D), being stabilized by van der Waals' contacts with the heme, PheB9(32), PheE15(62), ValG8(94), LeuG12(98), and IleH11(119) and by dipole-dipole interaction with the amide group of GlnE11(58) (Fig. 2D).

It has previously been shown that in Mt-trHbN the short and the long tunnel branches are separated by the two facing res-
The figure shows a cut view of the protein matrix tunnel. The figure was observed in two conformations (identified as residues PheE15(62) and LeuG12(98). In particular, PheE15(62) is found in double conformation in subunit B (closed conformation, in the A subunit. In contrast, residue PheE15(62) is present in only one conformation in subunit C. The first important movement of the dioxygen molecule along the tunnel path is represented by the simulation steps at 400–420 ps, with a transient (metastable) state at 410 ps. The main restricting role on the dioxygen molecule during the critical stages of transition from the short to the long tunnel branch is played by the side chains of PheB9(32), PheE15(62), and LeuG12(98). In view of the extent of side chain excursions, PheE15(62) is proposed as the main dioxygen gating residue (6).

**Discussion**

The structural investigations here reported show that xenon binding to three group I trHbs occurs along the protein matrix tunnel path previously identified (6, 9), following an ordered scheme. One xenon-binding site (Xe1 at 13.3–13.8 Å from iron) is conserved in all the three proteins as a high occupancy site. Mt-trHbN and Ce-trHb share a similar pattern of xenon-binding sites (Xe1, Xe2, and Xe5), despite very limited overall amino acid sequence identity (<20%). Moreover, the entry sites of the tunnel short and long branches are mapped by xenon binding in Mt-trHbN (Xe3 site) and in Ce-trHb (Xe6 site), respectively. Taken together, these observations point out that the xenon-binding sites outline experimentally, with perfectly comparable topology, the tunnel path proposed earlier, in the unbound versions of the different trHbs, as a group I trHb conserved structural feature (Fig. 2, A–C).

Butyl isocyanide binding to cyano-met Mt-trHbN also occurs along the identified tunnel path. Indeed, the aliphatic molecule is hosted in an extended conformation along the short tunnel branch in the A subunit. Incidentally, we notice that no coordination of the alkyl-isocyanide ligand to the heme iron atom could occur in Mt-trHbN, because the ligand diffusion experiment was based on crystals of the Mt-trHbN cyano-met form. Absence of butyl isocyanide coordination to the heme iron forces the ligand to explore the tunnel space, and might, in principle, prevent butyl isocyanide from residing in a specific location long enough to be detected through x-ray crystallography. Binding of butyl isocyanide in the tunnel is obviously stabilized by hydrophobic interactions. However, selection of the specific docking site within the tunnel span appears to be also guided by a dipolar interaction between the isocyanide moiety and GlnE11 (58) side chain. Although we have no direct explanation for the markedly different behavior in butyl isocyanide binding in the two Mt-trHbN subunits, it can be mentioned that the very weak electron density compatible with butyl isocyanide binding in the B subunit maps at the same binding site observed in the A subunit. Conformational differences between the two subunits, at the PheE15(62) residue had been noted earlier, in the oxygenated Mt-trHbN crystal structure (6).

The unrefined structural data mentioned for the Ce-trHb butyl isocyanide adduct are also fully in keeping with the above view. The fact that butyl isocyanide can bind to a site located next to the heme, while not exploiting iron coordination, indeed stresses the potential role of the trHb apolar tunnel in supporting diffusion (and accumulation) of low polarity molecules. It is worth noting that such low polarity molecules need not be simply diatomic (as shown by butyl isocyanide binding), or, if
diatomic, they may be hosted within the tunnel space in multiple copies (15).

The observation that fine variation of the protein matrix tunnel structure allows three group I trHbs to modulate xenon atom binding at a series of possible sites suggests that specific and diverse functional roles may be ascribed to the proteins considered. A recent study on Pc-trHb and Ce-trHb geminate and solvent phase CO rebinding processes has shown very unusual kinetic patterns, compared with other known hemoglobins and Mbs. In particular, the concentration dependence of the CO rebinding kinetics (two distinct phases at low CO concentration) indicates a role for the apolar tunnel and for the static/dynamic properties of the distal site hydrogen-bonding network in modulating reactivity (15).

Also within this respect, the lack of a sizeable tunnel space within the protein matrix of crystalline Ss-trHb (8) may be related to the many reported conformational readjustments affecting important residues in the distal cavity of the hexacoordinated protein (at sites B10, E7, E10, E11, and E14). Such conformational changes, as well as major backbone readjustments (e.g. shifts in the B and E helices), are related to the selection of residue E10(1His), instead of E7, for heme hexacoordination, and to the presence of a heme-HisH16(117) covalent link. In addition it could be considered that transition to an hexacoordinated heme may be facilitated by the availability of core cavities, allowing the five-coordinate protein species to achieve substantial conformational readjustments at a low energy cost. It is interesting to note that Ce-trHb, which also display heme hexacoordination at alkaline pH (25), is the group I trHb showing the largest tunnel volume, in its cyano-met form.

A network of monomeric globin structures shows that besides sperm whale Mb, where xenon cavities have long been recognized and mapped (10–14), structural conservation of the protein core cavities within the globin family is not an absolute rule. Within monomeric globin structures, small protein matrix cavities are occasionally observed; however, they do not match topologically those recognized in sperm whale Mb. It is also worth noting that if the trHb tunnel/cavities were exclusively the result of α-helix packing defects in the folded proteins, similar structural features should be recognizable also in conventional globins, sharing four main helices with the trHb fold. In this respect, group II trHbs are an interesting case, because group-specific residue substitutions (e.g. a Trp residue at the G8 site) are expected to affect the tunnel shape/volume substantially. Such a trend has been shown in the crystal structure of Mt-trHbO (9), where the only major cavity observed is the matching the Xe1 site, here shown to be a high occupancy of Mt-trHbO (9), where the only major cavity observed is the substantial. Such a trend has been shown in the crystal structure of the reaction matrix, endowed with more than one entry/exit points, is a structural feature that fully supports all of the above criteria, perhaps adding a structural environment around GluN11(45) to host the expected peroxynitrite (Fe3+−O−NO−) intermediate.

Substrate channeling is an acknowledged concept in different fields of enzymology (26–28). On the basis of the above reported data, we propose that, at least for Mt-trHbN, which displays an in vitro NO-dioxygenase activity (if supplied with a suitable reductase partner system), the term “ligand tunneling” may properly apply.

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