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Preliminary crystallographic studies of two C-terminally truncated copper-containing nitrite reductases from *Achromobacter cycloclastes*: changed crystallizing behaviors caused by residue deletion

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Abstract

The C-terminal segment of copper-containing nitrite reductase from *Achromobacter cycloclastes* (AcNiR) has been found essential for maintaining both the quaternary structure and the enzyme activity of AcNiR. C-terminal despentapeptide AcNiR (NiRc-5) and desundecapeptide AcNiR (NiRc-11) are two important truncated mutants whose activities and stability have been affected by residue deletion. In this study, the two mutants were crystallized using the hanging drop vapor diffusion method. Crystals of NiRc-5 obtained at pH 5.0 and 6.2 both belonged to the $P2_12_12_1$ space group with unit cell parameters $a = 99.0$ Å, $b = 117.4$ Å, $c = 122.8$ Å (pH 5.0) and $a = 98.9$ Å, $b = 117.7$ Å, $c = 123.0$ Å (pH 6.2). NiRc-11 was crystallized in two crystal forms: the tetragonal form belonged to the space group $P4_1$ with $a = b = 96.0$ Å and $c = 146.6$ Å; the monoclinic form belonged to the space group $P2_1$ with $a = 86.0$ Å, $b = 110.1$ Å, $c = 122.7$ Å, and $\beta = 101.9°$. The crystallizing behaviors of the two mutants differed from that of the native enzyme. Such change in combination with residue deletion is also discussed here.
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Nitrite reductase (EC 1.7.99.3) from *Achromobacter cycloclastes* (AcNiR) is a copper-containing protein that reduces nitrite to gaseous nitric oxide, resulting in the direct loss of fixed nitrogen from the terrestrial environment [1]. AcNiR is the first copper-containing nitrite reductase whose crystal structure has been analyzed [2]. Previous studies showed that AcNiR could be crystallized under a wide pH range from 5.0 to 6.8 and with the elevation of pH the crystal form will change from cubic to orthorhombic [3,4]. Crystal structure analysis [2,4] revealed that the enzyme was organized as a homo-trimer with each monomer comprising two Greek key β-barrel domains. A total of six copper atoms were found in the trimer, classified into two types: the type-1 copper (T1Cu) buried in each monomer is the site for the outer electron acceptor; the type-2 copper (T2Cu) located at the inter-subunit cleft is the site for nitrite binding and reduction. AcNiR is very stable in that it migrates as a trimer during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) without heating and reductant [5]. In fact, the trimer form of AcNiR has shown its rigid property under SDS detergent soaking even when the concentration is as high as 4% (w/v). A similar property has also been found in the copper-containing nitrite reductase from *Alcaligenes faeclis* [6] and *Bacillus halodenitrificans* [7] and it might be a common feature among such enzymes considering their highly conserved three-dimensional structure [8]. Based on the crystal structure, three factors are thought to determine the maintenance of AcNiR trimer: (1) the T2Cu to His306 bond; (2) the extensive intermonomer

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contact surface surrounding the T2Cu site; (3) C-terminal segment from residue 324–340, which extends from one monomer to another, mediating interaction between monomers [2]. Of these factors, the third is shown to be the most important one. In a previous study, we generated a series of C-terminally deleted AcNiR mutations to explore the possible role of the C-terminal tail in AcNiR. This study showed that, although far away from the active site, the C-terminal segment is essential for maintaining the enzyme activity as well as the rigid trimeric structure of AcNiR [5].

C-terminal despentapeptide AcNiR (NiRc-5) and desundecapeptide AcNiR (NiRc-11) are two important C-terminally deleted AcNiR analogues. After five residues (336–340) are deleted, NiRc-5 still remains a rigid trimer, shows typical native enzyme optical spectrum, but has lost about 30% activity. It is the first C-terminally deleted AcNiR analogue that begins to lose part of its enzyme activity. Furthermore, with 11 residues (330–340) deleted, NiRc-11 trimer could no longer resist the denaturation of sodium dodecyl sulfate (SDS) and lost its enzyme activity totally. It is the first C-terminally deleted AcNiR analogue to completely lose its enzyme activity. Therefore, these two mutations could be good candidates for studying the structural role of the C-terminal tail in maintaining the rigid trimeric structure and enzyme activity of AcNiR.

This paper reports on the crystallization and preliminary crystallographic studies of NiRc-5 and NiRc-11. The changed crystallizing behaviors of these two mutants caused by residue deletion are also discussed here.

Materials and methods

Molecular biology and protein purification. Cloning and expression of NiRc-5 and NiRc-11 genes and purification and biochemical analysis of the two proteins have been described in detail earlier [5,9]. Purified protein samples were freeze-dried and stored at −20 °C until used for crystallization.

Crystal growth. NiRc-5 and NiRc-11 were crystallized using the hanging drop vapor diffusion method, in which, typically, 2 μl protein solution was mixed with 2 μl reservoir solution and suspended on a coverslip over a reservoir containing 0.5 ml precipitant. The initial concentration of each protein solution was normally 10 mg/ml in distilled water. A variation of protein concentration between 7 and 15 mg/ml did not affect the crystal growth much. The crystallizing conditions for NiRc-5 were obtained by simulating native AcNiR. The reservoir solution consisted of 1.5 M ammonium sulfate, 0.2 M sodium chloride, and 0.1 M acetate or 2-morpholinoethanesulfonic acid monohydrate (MES) buffer. The incubating temperature was 25 °C. Large light-green crystals could appear under a wide pH range from 4.6 to 6.5 (buffered from acetate to MES) within one or two weeks (Figs. 1A and B). Sodium chloride was not essential for crystal growth, but as an additive, it helped to shorten the time and improve the reproducibility of crystal growth. A similar simulated search on NiRc-11 was unsuccessful. Thereafter, a sparse-matrix screen [10] using the Crystal Screen Kits I and II of Hampton Research was performed and this led to some useful results. The initial condition was then optimized until a reservoir solution composed of 16% polyethylene glycol 8000, 0.2 M ammonium sulfate, and 0.1 M MES buffer, pH 6.5, or 0.1 M sodium cacodylate buffer, pH 5.4, was found capable of yielding good NiRc-11 single crystals. It is important to note that the incubating temperature was critical for successful crystallization. When grown at 25 °C, NiRc-11 crystals seldom appeared even after several months' incubation, whereas crystals could grow up to a size of $0.15 \times 0.15 \times 0.15$ mm within a few days if placed at 32 °C. Further tests also showed that the NiRc-11 crystals could resist a temperature as high as 38 °C, but might be destroyed if placed below 15 °C for several hours. Crystals grown from the MES buffer system were all in the tetragonal pyramid form (Fig. 1C); while those grown from the cacodylate buffer system were in either monoclinic block form (Fig. 1D) or tetragonal pyramid form. The two crystal forms from the cacodylate buffer system appeared in the same well occasionally, but when the detergent: methyl-6-*o*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside (HECAMEG) was used as an additive, the block form crystals became dominant.

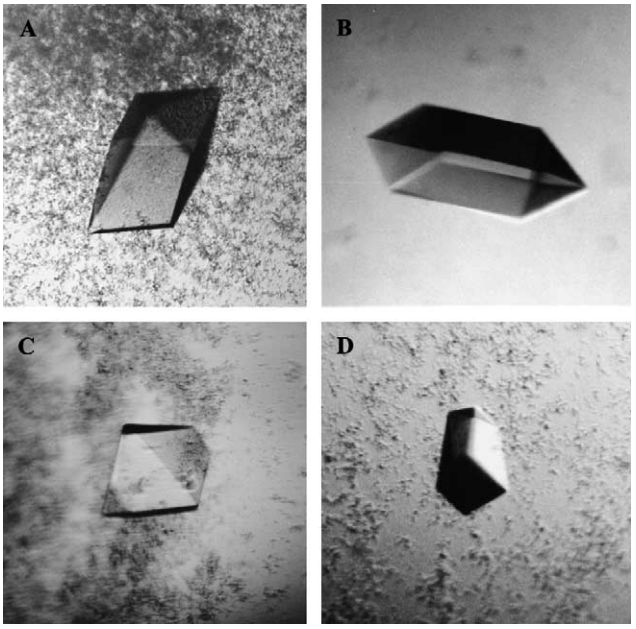

Fig. 1. Crystals of NiRc-5 and NiRc-11. (A) Orthorhombic crystal of NiRc-5 at acetate buffer pH 5.0. (B) Orthorhombic crystal of NiRc-5 at MES buffer pH 6.2. (C) Tetragonal crystal of NiRc-11 at MES buffer pH 6.5. (D) Monoclinic crystal of NiRc-11 at cacodylate buffer pH 5.4.

Data collection and processing. NiRc-5 crystals grown from pH 5.0 (buffered by acetate) and pH 6.2 (buffered by MES) were used for data collection. The pH 5.0 data set was collected at 100 K (Oxford Cryosystems) on a Mar345 imaging plate system at the National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences. A sealed tube X-ray generator operated at 40 kV and 50 mA was used to provide the Cu Kα radiation ($\lambda = 1.5418$ Å). The pH 6.2 data set was collected at 100 K (Oxford Cryosystems) with an R-AXIS IV imaging plate detector (Rigaku) on the BL6B synchrotron beamline at Photon Factory, Tsukuba, Japan. The wavelength of the X-rays used was 1.0 Å. Prior to flash-cooling, the crystals were dipped into an anti-freezing solution, which was the reservoir solution but enriched with 22% ethylene glycol as cryo-protectant.

For NiRc-11, data sets from two crystal forms were collected. A tetragonal form crystal grown from MES buffer, pH 6.5, was mounted in a thin-wall capillary and data were collected at room temperature on the Mar345 image plate system as described above. The data set of the monoclinic block form crystal was collected at 100 K using the same synchrotron facilities as applied to NiRc-5. The anti-freezing solution

Table 1
X-ray data-collection statistics

Data set	NiRc-5 (pH 5.0)	NiRc-5 (pH 6.2)	NiRc-11 (tetragonal)	NiRc-11 (monoclinic)
X-ray source	Cu Kα	PF (BL6B)	Cu Kα	PF (BL6B)
Wavelength (Å)	1.54	1.00	1.54	1.00
Temperature (K)	100	100	293	100
Space group	$P2_12_12_1$	$P2_12_12_1$	$P4_1$	$P2_1$
Unit cell parameters	$a = 99.0$ Å $b = 117.4$ Å $c = 122.8$ Å	$a = 98.9$ Å $b = 117.7$ Å $c = 123.0$ Å	$a = b = 96.0$ Å $c = 146.6$ Å	$a = 86.0$ Å $b = 110.1$ Å $c = 122.7$ Å $\beta = 101.9°$
Resolution range (Å) (outmost shell)	20–2.2 (2.25–2.20)	20–1.9 (1.94–1.90)	20–3.8 (3.89–3.80)	20–2.8 (2.86–2.80)
Observed reflections	435,939	451,920	64,881	181,604
Unique reflections	73,098 (4800)[a]	112,590 (7324)	13,067 (874)	51,545 (3004)
Completeness (%)	99.8 (99.3)	99.5 (98.5)	100 (100)	92.7 (80.1)
Mean $I/\sigma(I)$	13.4 (3.0)	14.5 (2.9)	9.6 (4.3)	27.2 (10.1)
R_{merge}[b] (%)	13.0 (58.6)	11.9 (52.9)	18.1 (42.5)	4.9 (14.9)

[a] Values in parentheses are for outer resolution shell.
[b] $R_{merge} = \Sigma_h\Sigma_i|I(h,i) - \langle I(h)\rangle|/\Sigma_h\Sigma_i I(h,i)$, where $I(h,i)$ is the intensity of ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

used here was 16% PEG8000, 0.1 M sodium cacodylate buffer, pH 5.4, 0.2 M ammonium sulfate, and 18% polyethylene glycol 400.

All the data sets were indexed, integrated, and scaled using the programs DENZO and SCALEPACK [11]. Statistics of data collection and processing are shown in Table 1.

Results and discussion

Data processing on NiRc-5 showed that crystals grown from either pH 6.2 or pH 5.0 belonged to the orthorhombic form, which is different from native AcNiR that will undergo a crystal form change from orthorhombic to cubic when the pH turns more acidic (below pH 6.0) [4]. The space group of the two crystals is $P2_12_12_1$ with unit cell parameters $a = 99.0$ Å, $b = 117.4$ Å, $c = 122.8$ Å (pH 5.0) and $a = 98.9$ Å, $b = 117.7$ Å, $c = 123.0$ Å (pH 6.2). Compared with native AcNiR orthorhombic crystals ($P2_12_12_1$, $a = 99.3$ Å, $b = 115.2$ Å, $c = 116.0$ Å), the NiRc-5 crystals exhibit a unit-cell volume expansion of approximately 7.6%. Assuming a calculated Matthews coefficient of $V_M = 3.54\,\text{Å}^3\,\text{Da}^{-1}$, there is one trimer in the asymmetric unit with a solvent content of 64.0% [12]. Data processing on the tetragonal form crystal of NiRc-11 suggested that it belongs to either the $P4_1$ or $P4_3$ space group. A preliminary rotation and translation function search using the Amore program [13] revealed that it most likely belonged to $P4_1$. It was also verified that, in this tetragonal crystal, there is one trimer in the asymmetric unit with a Matthews coefficient of $3.42\,\text{Å}^3\,\text{Da}^{-1}$ and a solvent content of 62.7%. The space group of the thick-block form NiRc-11 crystals was proved to be $P2_1$. With the use of the synchrotron source, the small crystal ($0.10 \times 0.2 \times 0.15$ mm) could give strong reflections of better than 2.8 Å. Assuming a calculated Matthews coefficient of $2.87\,\text{Å}^3\,\text{Da}^{-1}$, there are two trimers in the asymmetric unit with a solvent content of 55.5% in these monoclinic crystals.

The crystallizing behavior of NiRc-5 has changed in comparison with the native enzyme since it undergoes no crystal form change when the crystallizing pH varies. Crystallographic studies [4] of native AcNiR showed that, in the cubic form, the trimer is formed by three identical monomers, and C-terminal residues 335–340 of each monomer form six hydrogen bonds to residues 117–121 of the adjacent monomer. However, in the orthorhombic form, the trimer is partially desymmetrized in that residues 335–340 of chain A are moved away and point to the solvent region. Structure analysis revealed that such change resulted from crystal packing, in which N-terminal residues of symmetry-related chain A displaced residues A335–340 so as to complete new packing contacts in the orthorhombic crystals (Fig. 2). In contrast, the tail residues of chains B and C remain unchanged as they are not involved in the crystal packing. When the crystal form changes from cubic to orthorhombic, AcNiR has to overcome an energy barrier of breaking at least six hydrogen bonds for the removal of chain A's C-terminal residues. An elevation in crystallizing pH might contribute to such removal as derived from the crystallization of AcNiR. In addition, it can be proposed that orthorhombic crystals rather than cubic ones will appear preferentially if this packing barrier is eliminated. Deletion of residues 336–340 does demolish this energy barrier and as a result, only orthorhombic NiRc-5 crystals appear even when the pH drops to 4.6 during a pH-profile crystallizing condition screening. The slight expansion in unit cell volume of NiRc-5 crystals indicates that protein molecules in the unit-cell are arranged in a less compact way, which is consistent

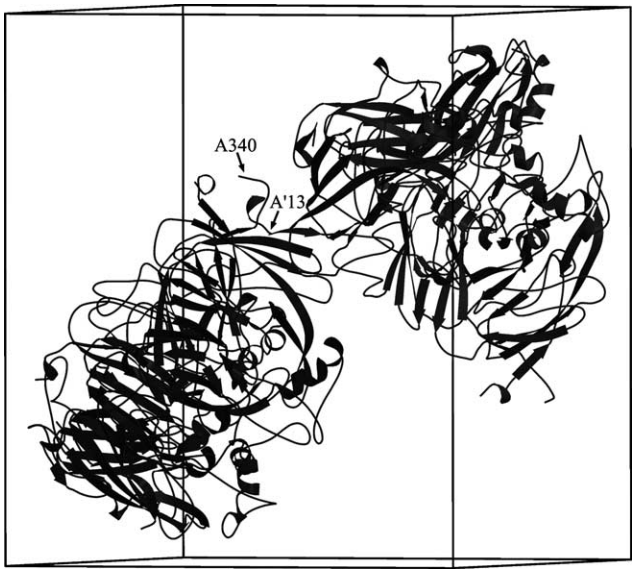

Fig. 2. Crystal packing near C-terminal segment of chain A in orthorhombic native AcNiR crystals. C-terminal residues of monomer A are displaced by N-terminal residues of the symmetry-related monomer A′ to form crystal-packing contacts. Figures are produced with programs Molscript [14] and Raster3D [15]. Coordinates are taken from Protein Data Bank with entry code 1NIA.

with the fact that orthorhombic crystals appear more easily after the five residues are deleted. As for the native enzyme, more compact packing might be needed to push the C-terminal residues of chain A aside.

After the deletion of 11 residues, NiRc-11 not only loses its enzyme activity totally but also changes in crystallizing condition significantly. Although NiRc-11 behaves as a monomer in SDS–PAGE without heating and reductant and even in solutions at a higher pH (>7.0) in gel filtration analysis (unpublished data), the packing unit of NiRc-11 crystals should still be a trimer considering its existence in the asymmetric unit. For the sake of successful crystal growth, it is best if the integrity of NiRc-11 trimer can be kept. Among the three factors determining the trimer maintenance previously mentioned, the third one has been impaired substantially by the deletion of 11 residues. As a result, the stability and other properties of the trimer are affected, which in turn, changes the crystallizing behavior of NiRc-11. The high-temperature dependent crystallization feature of NiRc-11 is unusual but might also be an internal need for successful crystal growth. Since the inter-subunit interactions mediated by the C-terminal tail are largely reduced, the maintenance of NiRc-11 trimer might rely much more on the hydrophobic interactions between two adjacent monomers. Based on the thermodynamic principle ($\Delta G = \Delta H - T\Delta S$), an elevation in temperature will enhance the hydrophobic interactions in protein [16]. Therefore, to form a rather rigid and homogeneous trimer for crystal packing, a moderately high temperature was selected as a key factor in crystal growth, so as to promote the initial nucleation and subsequent packing during the crystallization of NiRc-11.

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