Identification of Essential Catalytic Residues of the Cyclase NisC Involved in the Biosynthesis of Nisin*

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Nisin is a post-translationally modified antimicrobial peptide that has been widely used in the food industry for several decades. It contains five cyclic thioether cross-links of varying sizes that are installed by a single enzyme, NisC, that catalyzes the addition of cysteines to dehydroamino acids. The recent x-ray crystal structure of NisC has provided the first insights into the catalytic residues responsible for the cyclization step during nisin biosynthesis. In this study, the conserved residues His212, Arg280, Asp341, and Tyr285 as well as the ligands to the zinc in the active site (Cys284, Cys330, and His331) were substituted by site-directed mutagenesis. Binding studies showed that all mutants had similar affinities for NisA. Activity assays showed that whereas His212 and Asp341 were essential for correct cyclization as judged by the antimicrobial activity of the final product, Arg280 and Tyr285 were not. Mutation of zinc ligands to alanine also abolished the enzymatic activity, and these mutant proteins were shown to contain decreased levels of zinc. These results show that the zinc is essential for activity and support a model in which the zinc is used to activate the cysteines in the substrate for nucleophilic attack. These findings also argue against an essential role of Arg280 and Tyr285 as an active site general acid/base in the mechanism of cyclization.

Antimicrobial peptides containing nonproteinogenic amino acids are produced by numerous microorganisms (1). Many of these secondary metabolites are assembled by nonribosomal peptide synthesis. In addition, nature also employs an alternative strategy featuring the use of the ribosome to prepare the peptide backbone and subsequent posttranslational modification to increase the structural diversity of functional groups beyond the 20–22 proteinogenic amino acids (2). A common theme found in both products of NRPS and posttranslational modification is that they often contain macrocyclic structures to constrain their conformation and enhance their activity, specificity, and stability. Post-translationally modified peptides include microcins, such as the Escherichia coli antibiotic microcin B17 that contains the five-member heteroaromatic oxazoles and thiazoles (3), and the lantibiotics, a group of peptides containing unique intramolecular thioether linkages termed lantionines and methyllanthonines (4).

Nisin, the most prominent member of the lantibiotics, is highly effective (nanomolar minimum inhibitory concentrations) in inhibiting the growth of a broad spectrum of Gram-positive bacteria including food-borne pathogens and multidrug-resistant bacteria (5). The compound has been used extensively in the food industry as a preservative. Nisin exerts its high efficacy by pore formation in the membrane and disruption of cell wall biosynthesis. Both of these activities are mediated by binding to an indispensable cell wall biosynthetic intermediate, lipid II (6). Nisin contains five cyclic thioethers with ring sizes varying from four to seven amino acids (Fig. 1). These thioether cross-links are installed in lantibiotics by a two-step process that first involves dehydration of Ser and Thr residues to the corresponding dehydroalanines and dehydrobutyrines, followed by regioselective addition of Cys residues to the dehydroamino acids (Fig. 1). Recent in vitro studies on the dehydration step for the lantibiotic lactacin 481 have started to provide insights into the molecular mechanism of catalysis (7–9), but thus far little is known regarding the mechanism of the cyclization step of nisin biosynthesis. Previous work has shown that a single enzyme, NisC, is responsible for installing all five rings in nisin (10, 11), which are important for its biological activity. The opening or deletion of the rings, especially the A- and B-rings, results in a drastic decrease of biological activity (12–15). Indeed, these two rings in nisin make direct contacts with the pyrophosphate moiety of lipid II in an NMR structure of the 1:1 complex (16). The importance of the thioether cross-links has also been demonstrated for several other lantibiotics (15, 17, 18). Several previous studies have shown that cyclic lantionines also increase the stability and activity of non-lantibiotic peptides (19, 20), suggesting that this structural motif has much potential for engineering of bioactive peptides. To date, the utility of the lantionine conformational constraint has been underexplored, however, predominantly because chemical approaches to prepare lantionine-containing peptides, although powerful in synthesizing small peptides (21–25), are currently still challenging when applied to larger peptides with multiple ring structures (26, 27). Indeed, only one chemical synthesis of a lantibiotic, nisin, has been reported in the literature to date (28). Enzymatic cyclization is a promising alternative in this aspect, and lantibiotic biosynthetic enzymes appear to be good candidates for both reprogramming the structure of lantibiotics and introducing lantionines into non-lantibiotic peptides (15, 29, 30). A better understanding of the...

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mechanism of the cyclization step will provide important information for these bioengineering studies.

The x-ray structure of the nisin cyclase NisC displayed a zinc located at the mouth of an αααα-barrel with a tetrahedral coordination geometry (11). The ligands are made up of His331, Cys284, and Cys330, with a water molecule completing the coordination environment (Fig. 2). Other residues in the active site that are conserved in the LanC family of lantibiotic cyclases include His212, Asp141, Tyr285, and Arg280. In this study, the roles of the zinc ligands and active site residues of NisC were investigated by site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—The expression vector for His6-NisC, pET15b-NisC, was previously described (31). Plasmid DNA was prepared by using a QiAprep spin miniprep kit (Qiagen). Mutants were generated using the QuikChange method. The primer sequences were 5′-GGA TTA GCA AAT GGA CTT GCT GGA GTG GG-3′ (H212N), 5′-GGG ATT AGC ATT TGG ACT TGC TGG AGT GG-3′ (H212F), 5′-CAT TAG AGA TGC ATG GGC TTA TGG AGG TCC-3′ (C284A), 5′-CAT ATA TGA TGG CTC ATG GCT ATT CTG GTT TAA TAG AA-3′ (C330A), 5′-GGG AAG CAA GTT TCA TTA TGG AGT CAT GGT GCT ATG G-3′ (R280M), 5′-GGG AAG CAG TTT TCA TTA TGG AGT CAT GGT GCT ATG G-3′ (R280A), 5′-CAT TAC TCC TCC TGA TTA TAA CGT GAT TGA AGG TTT ATC-3′ (D141N), 5′-CAT TAC TCC TCC TGA TTA TAA CGT GAT TGA AGG TTT ATC-3′ (D141A), 5′-GAG ATG CAT GGT GCT TTG GAG GTC CAG GTA TTA G-3′ (Y285F), 5′-GAG ATG CAT GGT GCG CTG GAG GTC CAG GTA TTA G-3′ (Y285A), 5′-GAT TCA TAT ATG ATT TGC GCT GGC TAT TCT GG-3′ (H331A), and the corresponding reverse complementary fragments. All mutants were verified by DNA sequencing.

**Overexpression and Purification of NisC Mutants**—A 20-ml culture of E. coli BL21 (DE3) cells carrying the NisC mutant constructs was grown in LB amp at 37 °C overnight with shaking. An aliquot of 10 ml of overnight culture was added to 1 liter of LB amp and grown at 37 °C with shaking to an A600 of 0.7–0.9. Overexpression of the protein was induced with 0.2 mM isopropyl β-D-thiogalactoside, and the bacteria were grown in LB amp (supplemented with 100 μM ZnCl2) at 18 °C for 15–20 h. The cells were harvested by centrifugation and resuspended in 15 ml of resuspension buffer (20 mM Tris, 0.5 M KCl, 10% glycerol, pH 8.3) containing protease inhibitor mixture (Roche Applied Science). Cells were sonicated, and the cellular debris was removed by centrifugation at 14,000 rpm for 30 min. The supernatant was filtered and applied to a cobalt affinity column (Cobalt Talon; BD Biosciences). The column was washed with 15 column volumes of equilibration buffer (20 mM Tris, 0.5 M NaCl, 10% glycerol, pH 7.5) and 5 column volumes of wash buffer (20 mM Tris, 0.5 M NaCl, 20 mM imidazole, 10% glycerol, pH 7.5). NisC mutant proteins were eluted with 5 column volumes of elution buffer (20 mM Tris, 0.5 M NaCl, 150 mM imidazole, 10% glycerol, pH 7.5). Elution fractions were monitored by Bradford protein assay (Bio-Rad) and SDS-PAGE. The fractions containing NisC mutant were combined and concen-
trated with Amicon-12 (YM-10; Millipore). The concentrated protein was desalted with a PD-10 desalting column (GE HealthCare) and stored at −80 °C. Protein concentration was measured using the BCA assay (Pierce).

Removal of His Tag with Thrombin—The cleavage of the His tag was performed by incubating the His6-NisC (wild type or mutant) with biotinylated thrombin (Novagen) overnight at 4 °C. Biotinylated thrombin was then removed with streptavidin-agarose beads (Novagen). The resulting protein was purified by cobalt affinity chromatography on a fast flow column (BD Bioscience).

Metal Analysis—All flasks and beakers used were prerinsed with 20% HNO3 and Milli-Q water. Dialysis was performed with Slidealyzers (Pierce) presoaked in 20 mM Tris, pH 7.5, containing 10 g/liter Chelex-100 (Bio-Rad). NisC (wild type or mutant) was dialyzed against four changes of 20 mM Tris, 300 mM NaCl, pH 7.5, with 25–50 g/liter Chelex-100 in the beaker, over a total time of 48 h at 4 °C. The Chelex-100-treated protein was then concentrated with an Amicon-4 ultrafiltration set-up (YM-10; Millipore) that had been presoaked in 20 mM Tris, pH 7.5, containing 10 g/liter Chelex-100. Metal content was determined by inductively coupled plasma MS (ICP-MS) at the University of Illinois at Urbana-Champaign. The zinc content was also analyzed using a spectroscopic assay based on the absorption change at 500 nm associated with the formation of a zinc complex with 4-(2-pyridylazo)resorcinol (PAR2; Sigma) as previously described (31).

Activity Assay—Dehydrated prenisin was overexpressed and purified as previously described (11). A sample of 3 μl of 2 mg/ml dehydrated prenisin (final concentration ~21 μM) was mixed with 5 μl of 10× assay buffer (final concentration: 25 mM Tris, 2.5 mM MgCl2, pH 7.0) and 2.5 μl of 20× ZnCl2 (final concentration 5 μM). Then NisC was added (final concentration of wild type NisC was 1 or 10 μM, and final concentration of NisC mutants was 10 μM). The total volume of the assay was brought up to 50 μl with distilled sterile water. The reaction mixture was incubated at room temperature for 1.5 h. A sample of 1 μl of 4.5 μM trypsin was added to each assay sample (final concentration ~0.2 μM), and the mixtures were incubated at room temperature for 3 h. The resulting mixtures were checked by MALDI-TOF MS, and the proteolytic fragment corresponding to mature nisin was observed. Calcd.: 3354. Found: 3355 M + H. Subsequent bioassays were performed as previously described (11).

RESULTS

Constructs containing the nisC mutant genes encoding for C284A, C330A, H331A, D141A, D141N, R280A, H212N, H212F, R280M, W283H, W283A, Y285F, and Y285A were generated using the QuikChange protocol. The corresponding proteins were heterologously overexpressed in E. coli as histidine-tagged proteins. All mutants except W282H, W282A, and D141A could be purified to >95% homogeneity using Co2+ affinity chromatography. The D141A mutant was produced in the form of insoluble inclusion bodies, and both Trp mutants, while expressed in soluble form, could not be purified without precipitation during metal affinity chromatography. As a result, no purified protein was obtained. All purified proteins were analyzed by circular dichroism and displayed very similar spectra (see supplemental material). Furthermore, all mutants bound NisA that was fluorescently labeled at the N terminus with similar affinities as wild type NisC (see supplemental material).

The mutant proteins in which a metal ligand was substituted or that did not show activity (see below) were analyzed by two methods for their zinc content. The proteins expressed in cells grown on media supplemented with 100 μM ZnCl2 were analyzed by ICP-MS after removal of the His6 tag and exhaustive dialysis against chelex-treated buffer. In addition, a spectrophotometric assay using the metallochromic indicator PAR (32) was used to verify the metal content. In this method, the proteins were treated with guanidine hydrochloride and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to trap reactive cysteines as disulfides as previously described (31), and after the addition of PAR, the orange Zn(PAR)2 complex was observed and quantified by the absorbance at 500 nm. For proteins that contained nearly stoichiometric quantities of zinc, both approaches resulted in similar results. Mutants with a very low content of zinc as determined by the PAR assay were not analyzed by ICP-MS, since it would require large amounts of protein, given the limit of detection of the available instrumentation in this study. The data in Table 1 show that the relative zinc content of the zinc ligand mutants is significantly decreased, whereas the other mutants have zinc levels comparable with the wild type NisC, which contains one zinc per polypeptide (11, 31).

To date, the LanB dehydratase activity, including that of NisB, has not been reconstituted successfully in vitro (33, 34). Until recently, the lack of a functional NisB prevented in vitro characterization of the cyclization reaction. This hurdle was overcome by using a recently reported engineered Lactococcus lactis strain that carries a plasmid encoding the NisA, NisB, and NisT proteins but that lacks the genes for the NisC and NisP enzymes (35). The resulting strain was found to secrete the 8-fold dehydrated NisA peptide. In the current work, this organism was grown on a 3–5-liter scale, and the cells were then removed by centrifugation. Precipitation of all proteinaceous materials in the supernatant with trichloroacetic acid followed by resuspension of the pellet and purification by either high pressure liquid chromatography or a C18 Sep-Pak cartridge allowed isolation of the 8-fold dehydrated peptide. In the puri-

| NisC     | Relative zinc content | Activity |
|----------|-----------------------|----------|
| Wild type| 1.00*                 | +        |
| C284A    | 0.05                  | −        |
| C330A    | 0.04                  | −        |
| H331A    | 0.14                  | −        |
| H212N    | 1.36                  | −        |
| H212F    | 1.11                  | −        |
| D141N    | 1.03                  | −        |
| H331N    | 0.14                  | −        |

* Wild type NisC contained 0.87 ± 0.10 eq of zinc/polypeptide. This value is the average of two PAR assays and ICP-MS analysis.

2 The abbreviations used are: PAR, 4-(2-pyridylazo)resorcinol; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ICP, inductively coupled plasma; MS, mass spectrometry.
Mutagenesis of the Nisin Cyclase

![Image](60x504 to 288x733)

FIGURE 3. Antimicrobial activity of the assay products obtained from incubation of dehydrated NisA with NisC and NisC mutants and subsequent treatment with trypsin. The indicator strain was *L. lactis* NZ9000. The proteins used were as follows. A, 1 μM NisC (lane 1), 10 μM NisC (lane 2), 10 μM H212N-NisC (lane 3), 10 μM H212F-NisC (lane 4), 10 μM R280A-NisC (lane 5), 10 μM R280M-NisC (lane 6), no enzyme (lane 7), and cell-free spent medium of nisin producer strain (lane 8). B, 1 μM NisC (lane 1), 10 μM NisG (lane 2), 10 μM D141N-NisC (lane 3), 10 μM C284A-NisC (lane 4), 10 μM C330A-NisC (lane 5), no enzyme (lane 6), and cell-free spent medium of nisin producer strain (lane 7). Commercial nisin (nisaplin) is shown in lane 6. C, His6-NisC (10 μM; lane 1), wild type NisC (10 μM) treated with Chelex-100 (lane 2), Y285A-NisC (10 μM; lane 3), Y285F-NisC (10 μM; lane 4), H331A-NisC (10 μM; lane 5), no enzyme (lane 6), and cell-free broth of nisin producer strain (lane 7). Lane 8 contains trypsin at an amount utilized in the cleavage of leader peptide.

FIGURE 4. Proposed mechanism for the cyclization reaction catalyzed by NisC illustrated for the formation of the B-ring of nisin.

To test the cyclization activity of the NisC mutants, the dehydrated prenisin peptide was treated with each of the NisC mutants. The cyclization reaction does not bring about a change in molecular weight, and therefore routine mass spectrometry cannot be used to analyze the reaction. We therefore turned to an antimicrobial bioassay after removal of the leader peptide by a substoichiometric amount of trypsin. We note that use of a bioactivity assay to investigate the cyclization reaction is a very stringent test, since it probes not only the formation of the rings but also determines whether they are formed with the correct regio- and stereochemistry. This stringency is important, because nonenzymatic cyclization of cysteine residues onto dehydroamino acids is a rather facile process (21–23), but in the case of dehydrated prenisin, the nonenzymatic reaction is a very stringent test, since it probes not only the formation of the rings but also determines whether they are formed with the correct regio- and stereochemistry. This stringency is important, because nonenzymatic cyclization of cysteine residues onto dehydroamino acids is a rather facile process (21–23), but in the case of dehydrated prenisin, the nonenzymatic reaction does not produce bioactive compound (11, 26, 35). The assay mixtures after trypsin treatment were checked by MALDI-TOF MS, and the proteolytic fragment corresponding to mature nisin was observed. Trypsin removes the leader peptide by proteolysis C-terminal to Arg; no peaks corresponding to cleavage within mature nisin was observed, presumably because Lys and Lys are adjacent to methyllanthionine rings, which deactivate trypsin cleavage. The resulting mixtures were applied to a lawn of bacteria of the nisin-sensitive strain *L. lactis* NZ9000.

All NisC mutants were analyzed using the protocol described above. The H212N and H212F mutants were not able to convert the dehydrated NisA peptide into bioactive nisin (Fig. 3A). On the other hand, a clear zone of inhibition was observed with the assay products resulting from incubation of dehydrated NisA with R280A and R280M and subsequent trypsin treatment. Although not quantitative, the size of the inhibition zone was comparable with that of wild type NisC (Fig. 3A), suggesting that these mutants had similar activity as the wild type enzyme and that Arg is not essential for the cyclization activity of NisC. In contrast, replacement of the zinc ligands Cys, Cys, and His by Ala resulted in mutants that were not able to process the dehydrated prenisin peptide and produced a bioactive compound after the removal of the leader peptide (Fig. 3, B and C). Similar behavior was observed for D141N and Y285A, but Y285F did consistently show the production of bioactive nisin.

DISCUSSION

The recent crystal structure of the nisin cyclase NisC revealed the first insights into the mechanism that the LanC enzymes may utilize to introduce the characteristic lanthionine and methyllanthionine rings into lantibiotics. Based on the x-ray structure, we postulated a working model in which the zinc functions as a docking site for the cysteines of the substrate (11). The rate constant for the addition of free thiols to β, β-unsaturated centers is 10-fold smaller than observed for the corresponding thiolates (37); hence, substrate deprotonation is required for lanthionine formation at neutral pH. Metal coordination achieves a lowering of the pKa of the thiols and may also orient the resulting thiolate for regioselective nucleophilic attack onto the dehydroalanine (Dha) or dehydrobutyrine (Dhb) residues (Fig. 4). The zinc site in other enzymes that alkylate thiols (38) is thought to have a finely balanced coordination environment that on the
His212 to a water molecule that was liganded to zinc in the thiolates in the model (Fig. 4). Based on the proximity of varying sets of donor ligands (42–52). Indeed, upon sub-eral model studies on small molecule metal complexes with fur donors to the catalytic zinc has been established in several proteins. In this scenario, this residue might be the active site base that deprotonates the cysteines that coordinate to the zinc. In this case, Arg280 is not essential, it sheds doubt on a role as an active site general acid that protonates the enolate, leaving Tyr285, His212, or water to fulfill this role. Like Arg280, Tyr285 is not conserved in the cyclization reaction. Similar results were very recently reported in a study on the biosynthesis of the lantibiotic subtilin in which the zinc ligands of the subtilin dehydrated NisA substrate, supporting an essential role for the active site base for deprotonation of the thiol substrate is present in the crystal structures of farnesyl transferase (61), methionine synthase (62, 63), and betaine-homocysteine methyltransferase (56). Indeed, betaine-homocysteine methyltransferase (57), cobalamin-independent Met synthase (58, 59), and epoxynylkane: CoM transferase (60). In all cases except the Ada protein, which contains four Cys ligands, mutations of one of the zinc ligands resulted in proteins with depleted amounts of Zn2+.

The current work also sheds light on the importance of the other active site residues in NisC. Arg280 is not essential for cyclization, since both R280A and R280M were able to produce active nisin. These results agree with the recent in vivo mutagenesis of SpaC in which mutation of the corresponding Arg299 to Ala in Bacillus subtilis ATCC6633 did not abolish subtilin production (53). The finding that Arg280 is not critically important for cyclization activity may also explain why this residue, although completely conserved in the currently reported LanM proteins, is absent in the related LanM lantibiotic synthetases that contain a C-terminal domain with low but detectable sequence identity with the LanC proteins (Fig. 5). If Arg280 is not essential, it sheds doubt on a role as an active site general acid that protonates the enolate, leaving Tyr285, His212, or water to fulfill this role. Y285F-NisC showed activity in the in vitro cyclization assay, whereas Y285A did not. The latter result is consistent with in vivo mutagenesis of SpaC involving the corresponding tyrosine (Tyr304) (53); the Y304F-SpaC mutant was not reported. The activity of the tyrosine to phenylalanine mutant of NisC suggests that the presence of an aromatic residue is critical but it argues against the involvement of this residue as active site acid. Like Arg280, Tyr285 is not conserved in the cyclization domain of the LanM proteins (Fig. 5).

The data obtained with the mutants of Arg280 and Tyr285 leave the question of the identity of the active site acid that protonates the enolate intermediate. Although our data do not rule out the possibility that a water molecule fulfills this role, the findings reported here lead us to reevaluate the proposed role of His212 as a general base that deprotonates the thiol of the substrate. It is noteworthy that no such dedicated active site base for deprotonation of the thiol substrate is present in the crystal structures of farnesyl transferase (61), methionine synthase (62, 63), and betaine-homocysteine methyltransferase (64). Indeed, the pK_a of the cysteine residue of farnesyl transferase is depressed to about 6–7 upon metal coordination (39–41), indicating that an enzymatic base may not be essential. However, our mutagenesis studies show that both His212 and Asp214 that is hydrogen-bonded to His212 in the crystal structure of NisC are required for correct cyclization. Mutation of the corresponding His in the subtilin cyclase SpaC (His231) to Ala also aborted the in vivo production of subtilin. Thus, it is certainly possible that this pair is involved in the protonation of the enolate or altern-
Mutagenesis of the Nisin Cyclase


tively, in the electrophilic activation of the carbonyl group of the dehydroamino acids.

The final residue that is not a zinc ligand that was mutated in this study is Trp\textsuperscript{283}. This amino acid is conserved in both the LanC and LanM proteins (Fig. 5) and is located close to the active site in the NisC structure (Fig. 2). Based on the mutagenesis studies presented here, this residue is important for the stability of the protein, since mutants at this position were prone to precipitation and could not be purified. Whether it also plays a role in catalysis could therefore not be investigated in this study. Mutation of the analogous Trp\textsuperscript{302} in SpaC in a subtilisin-producing strain also abolished lantibiotic production, confirming the importance of the residue (53).

In summary, this study shows that mutation of the ligands to the zinc in lantibiotic cyclases abolishes correct macrocyclization of the dehydrated NisA substrate. The results presented also indicate that His\textsuperscript{212} and Asp\textsuperscript{141} are critical to control cyclization, whereas Arg\textsuperscript{280} and Tyr\textsuperscript{285} are not absolutely required. Definitive assignment of the role of His\textsuperscript{212} as either general acid or general base will require a co-crystal structure of the substrate or substrate analog with the enzyme. The mutants reported herein may facilitate obtaining such a structure.

REFERENCES

1. Walsh, C. T. (2003) Antibiotics: Actions, Origins, Resistance, American Society for Microbiology Press, Washington D.C.
2. Jack, R. W., and Jung, G. (2000) Curr. Opin. Chem. Biol. 4, 310–317
3. Chiou, S. J., Riordan, C. G., and Rheingold, A. L. (2003) J. Biol. Chem. 278, 4571–4575
4. Morlok, M. M., Janak, K. E., Zhu, G., Quarless, D. A., and Parkin, G. (2005) Inorg. Chem. 44, 13613–13624
5. Melnick, J. G., Zhu, G., Buccella, D., and Parkin, G. (2006) J. Am. Chem. Soc. 128, 11596–11603
6. Okeley, N. M., Zhu, Y., and van der Donk, W. A. (2000) J. Med. Chem. 43, 3746–3754
7. Okeley, N. M., Zhu, Y., and van der Donk, W. A. (2000) Org. Lett. 2, 3603–3606
8. Zhou, H., and van der Donk, W. A. (2002) Org. Lett. 4, 1335–1338
9. Helfrich, M., Entian, K.-D., and Stein, T. (2007) J. Inorg. Biochem. 101, 1147–1154
10. Meldrum, B., and Vahrenkamp, H. (2005) Biochemistry 44, 969–978
11. Parkin, G. (2004) Chem. Rev. 104, 699–767
12. Morlok, M. M., Janak, K. E., Zhu, G., Quarless, D. A., and Parkin, G. (2005) J. Am. Chem. Soc. 127, 14039–14050
13. Ibrahim, M. M., Seebacher, J., Steinfeld, G., and Vahrenkamp, H. (2005) Inorg. Chem. 44, 8531–8538
14. Helfrich, M., Entian, K.-D., and Stein, T. (2007) Biochemistry 46, 3224–3233
15. Harris, C. M., and Poulter, C. D. (2002) Biochemistry 41, 10554–10562
16. Sun, L. J., Yim, C. K., and Verdine, G. L. (2001) Biochemistry 40, 11596–11603
17. Goulding, C. W., and Matthews, R. G. (1997) Biochemistry 36, 15749–15757
18. Osapay, G., Prokai, L., Kim, H. S., Medzihradzkys, K. F., Coy, D. H., Liapakis, G., Reisine, T., Melacini, G., Zhu, Q., Wang, S. H., Mattern, R. H., and Goodman, M. (1997) J. Med. Chem. 40, 2241–2251
19. Chen, P., Novak, J., Kirk, M., Barnes, S., Qi, F., and Caufield, P. W. (1998) Appl. Environ. Microbiol. 64, 2335–2340
20. Bycroft, B. W., and Roberts, G. C. K. (1996) J. Biol. Chem. 271, 115749–115757
21. Okeley, N. M., Zhu, Y., and van der Donk, W. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 100, 3695–3700
22. Per, E. J. (2002) Curr. Opin. Chem. Biol. 6, 969–978
57. Breksa, A. P., III, and Garrow, T. A. (1999) Biochemistry 38, 13991–13998
58. González, J. C., Peariso, K., Penner-Hahn, J. E., and Matthews, R. G. (1996) Biochemistry 35, 12228–12234
59. Zhou, Z. Z., Peariso, K., Penner-Hahn, J. E., and Mathews, R. G. (1999) Biochemistry 38, 15915–15926
60. Krum, J. G., Ellsworth, H., Sargeant, R. R., Rich, G., and Ensign, S. A. (2002) Biochemistry 41, 5005–5014
61. Park, H. W., Boduluri, S. R., Moomaw, J. F., Casey, P. J., and Beese, L. S. (1997) Science 275, 1800–1804
62. Pejchal, R., and Ludwig, M. L. (2005) PLoS Biol. 3, e31
63. Evans, J. C., Huddler, D. P., Hilgers, M. T., Romanchuk, G., Matthews, R. G., and Ludwig, M. L. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3729–3736
64. Evans, J. C., Huddler, D. P., Jiracek, J., Castro, C., Millian, N. S., Garrow, T. A., and Ludwig, M. L. (2002) Structure 10, 1159–1171