Specific and Potent Inhibition of NAD\(^+\)-dependent DNA Ligase by Pyridochromanones*

Received for publication, June 18, 2003, and in revised form, July 15, 2003
Published, JBC Papers in Press, July 15, 2003, DOI 10.1074/jbc.M306479200

Heike Brötz-Oesterhelt‡‡, Igor Knezevic†, Stephan Bartel†, Thomas Lampe‡, Ute Warnecke-Eberz†, Karl Ziegelbauer‡, Dieter Häbich†, and Harald Labischinski‡

From the ‡Department of Anti-infectives and §Department of Chemistry, Bayer AG, Bayer Health Care, Pharma Research, Aprather Weg 18a, D-42096 Wuppertal, Germany.

Pyridochromanones were identified by high throughput screening as potent inhibitors of NAD\(^+\)-dependent DNA ligase from Escherichia coli. Further characterization revealed that eubacterial DNA ligases from Gram-negative and Gram-positive sources were inhibited at nanomolar concentrations. In contrast, purified human DNA ligase I was not affected (IC\(_{50}\) > 75 \(\mu\)M), demonstrating remarkable specificity for the prokaryotic target. The binding mode is competitive with the eubacteria-specific cofactor NAD\(^+\), and no intercalation into DNA was detected. Accordingly, the compounds were bactericidal for the prominent human pathogen Staphylococcus aureus in the low \(\mu\)g/ml range, whereas eukaryotic cells were not affected up to 60 \(\mu\)g/ml. The hypothesis that inhibition of DNA ligase is the antibacterial principle was proven in studies with a temperature-sensitive ligase-deficient E. coli strain. This mutant was highly susceptible for pyridochromanones at elevated temperatures but was rescued by heterologous expression of human DNA ligase I. A physiological consequence of ligase inhibition in bacteria was massive DNA degradation, as visualized by fluorescence microscopy of labeled DNA. In summary, the pyridochromanones demonstrate that diverse eubacterial DNA ligases can be addressed by a single inhibitor without affecting eukaryotic ligases or other DNA-binding enzymes, which proves the value of DNA ligase as a novel target in antibacterial therapy.

Multiple drug resistance among bacterial pathogens is spreading even in developed countries and has made many currently available antibiotics ineffective (1). As a consequence the number of reports on therapy failures increases and treatment costs rise, causing a growing public health problem. Thus, the search for novel antibacterial classes with innovative mechanisms of action is crucial to keep pace with the innate adaptability of the bacterial population. From the information revealed by sequencing more than 80 bacterial genomes, many novel target ideas have emerged in the last decade. However, even classical target areas such as cell wall, protein, or DNA synthesis contain many vital reactions not exploited in antibacterial therapy so far.

DNA ligases are promising target candidates because they are indispensable for many fundamental processes in DNA metabolism including the linkage of Okazaki fragments during replication, recombination processes, and repair pathways requiring resynthesis of DNA (2, 3). Their crucial function is emphasized by the fact that eukaryotic cells contain several isoenzymes and that viruses encode their own ligases (3, 4).

The reaction catalyzed by the DNA ligases, the joining of DNA and finally released when the ligase catalyzes the attack by the active site lysine on the AMP moiety of a cofactor, resulting in a covalent enzyme-AMP intermediate. The AMP is then transferred to the 5’-phosphate end of the nicked duplex DNA and finally released when the ligase catalyzes the attack by the adjacent 3’-hydroxyl group during the formation of a phosphodiester bond.

One aspect in considering DNA ligase as a potential antibacterial target is the distribution and homology among major bacterial pathogens. Eubacterial DNA ligases are extensively conserved over the entire length of the polypeptides (5). In contrast, if eubacterial and eukaryotic representatives are compared, there is little sequence homology apart from a short KXDG motif around the active site lysine. However, recent investigations reveal structural similarities especially in the AMP-binding region (3, 5).

Different cofactor requirements between the distinct ligase families raise the chance to find specific inhibitors directed exclusively against the eubacterial enzymes. Although the cofactor of eukaryotic, archaeal, and viral DNA ligases is ATP, which is degraded to AMP and pyrophosphate in the course of ligation, all eubacteria possess a ligase which uses NAD\(^+\) for this purpose, the final products being AMP and NMN (3, 5). In studies with temperature-sensitive or deletion mutants NAD\(^+\)-dependent DNA ligases were shown to be essential for survival in several bacterial species (e.g. LigA in Escherichia coli (6), YerG in Bacillus subtilis (7), and Lig in Staphylococcus aureus (8)).

For human DNA ligase I several natural product inhibitors have been described which mostly intercalate into DNA and which were evaluated for their potential as antitumor agents (9–11). In contrast, there is only one report on compounds targeting a eubacterial DNA ligase with some specificity. Ciarrocchi et al. (12) demonstrated that derivatives of the antimalaria drug chloroquine inhibited the E. coli DNA ligase LigA with IC\(_{50}\) values down to the single digit micromolar range. However, human DNA ligase I and the ligase of T \(_2\)-bacteriophage were also affected at 10-fold higher concentrations (12).

Here we present the pyridochromanones as a novel class of potent DNA ligase inhibitors. The compounds inhibited the purified NAD\(^+\)-dependent enzymes from both E. coli and Streptococcus pneumoniae in the nanomolar concentration range.
Specific Inhibition of NAD+-dependent DNA Ligase

This is to our knowledge the first demonstration of an inhibitory activity against a DNA ligase from a Gram-positive bacterium. Although the pyridochromones interfere broadly with eubacterial DNA ligases, human DNA ligase I is not inhibited up to 75 μM demonstrating that it is possible to discriminate between NAD+ and ATP-dependent representatives with high specificity.

MATERIALS AND METHODS

Antibacterial—Pyridochromones—Pyridochromones were synthesized at Bayer according to a previously described procedure (13). Ciprofloxacin was also prepared at Bayer, and novobiocin was obtained from Sigma-Aldrich.

Measurement of E. coli DNA Ligase Activity via Detection of Amp Release—In this assay format we detected the Amp released by E. coli DNA ligase LigA upon ligation of oligonucleotides. Adenylate kinase and pyruvate kinase were used to convert the AMP to ATP, which was quantified by the luminescence generated by ATP-dependent firefly luciferase. The nicked duplex template for DNA ligase was obtained by digesting and purifying pBR322 in 20 m M Tris/HCl, pH 8.0, 10 mM (NH4)2SO4, 1 mM EDTA, 10 mM NaCl, and 1 mM DTT and solubilized in the same buffer. For the supercoiling assay 100 ng of relaxed pUC18 DNA was incubated with 2 unit of Micrococcus luteus DNA gyrase (Invitrogen), 35 mM Tris/HCl (pH 7.5), 20 mM KCl, 0.1 mM EDTA, 10 mM mercaptoethanol, 10% (w/v) glycerol, 2 mM spermidine (Sigma-Aldrich), 1 mM ATP, and 0.002% (w/v) BSA for 80 min at 37 °C and analyzed in a 1% agarose gel.

Gel Shift Assay—200 ng of plasmid DNA (pBlueScript SK+; Stratagene) was incubated with increasing inhibitor concentrations in TE buffer for 30 min at room temperature. Subsequently, the DNA was analyzed in a 1% agarose gel. DAPER dye (Pierce) was used as a positive control.

Ethidium Bromide Displacement Assay—This assay measured the DNA intercalating properties of a compound by its ability to compete with ethidium bromide for DNA binding. Detection of ethidium bromide displacement from DNA is based on the strong loss in fluorescence that occurs upon its detachment from the double helix (15). The assay mixture contained in a volume of 100 μl 0.6 μg of S. aureus DNA (Invitrogen), 5 μM ethidium bromide, 35 mM Tris/HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA. Upon addition of the inhibitor in increasing concentrations the fluorescence of ethidium bromide was immediately detected at an excitation wavelength of 485 nm and an emission wavelength of 512 nm.

Heterogenous Expression of Human DNA Ligase I and S. aureus DNA Ligase in an E. coli ligA Mutant—The recombinant plasmid pBlueScript SK−:::ligI 250–919 containing the catalytic domain of human DNA ligase I (14) was transformed into E. coli GR501 carrying the chromosomal temperature-sensitive ligA mutation lig251Δ6 (6) The S. aureus ligI gene was amplified from the genome of S. aureus 133 (determined by the DSM strain collection, Braunschweig, Germany under the number DSM 11832). Primers were derived from the sequence determined by the Institute for Genomic Research for S. aureus COL (tigr.org). The PCR product was cloned into SacII and XhoI-digested pBlueScript SK+ (Stratagene) and transformed into E. coli GR501. As GR501 encoded no lac repressor, expression of the proteins from pBlue- script SK−:::ligI occurred constitutively. In growth experiments the strains expressing the human or S. aureus DNA ligases were compared with a control GR501 strain carrying empty pBlueScript SK− without gene insertion. All strains were grown in Iso-sensitest broth (Oxoid) at 28 or 37 °C. For the control strain GR501 28 °C represents the permissive and 39 °C the restrictive temperature. At 37 °C growth was already delayed. Propagation of the plasmids was ensured by the addition of 50 μg/ml ampicillin to the overnight culture. In the actual growth experiments the antibiotic was omitted.

Determination of Antimicrobial Activity—MIC values were determined in broth microdilution assays in microtiter plates in a volume of 200 μl. Serial 2-fold dilutions of antibacterial compounds were seeded into 100 μl of broth in the case of C. albicans and B. subtilis 168 trpC2 (16), of 107 CFU/ml in the case of E. coli GR501, and of 106 CFU/ml in the case of Candida albicans ATCC 200498. Bacteria were grown in Iso-sensitest broth and C. albicans in yeast nitrogen base broth (Difco), the latter being supplemented with 7.2 g of Na2HPO4, 3.55 g of KH2PO4, and 10 g of glucose/liter of medium. After incubation for 18 h for both strains or 24 h for C. albicans at 37 °C in ambient air, MICs were read as the lowest concentrations of compounds that prevented visible microbial growth. In the case of E. coli the medium contained 25 μg/ml polymyxin B nonapeptide (Sigma-Aldrich) to facilitate permeation of the inhibitors across the outer membrane.

1 The abbreviations used are: BSA, bovine serum albumin; TBS, Tris-buffered saline; CFU, colony-forming units; DAPI, 4’-6-diamidino-2-phenylindole; MIC, minimal inhibitory concentration; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; TE, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA.
Time-kill Studies—An exponentially growing culture of *S. aureus* 133 in Isosensitest broth was treated at an *A*<sub>600</sub> of 0.2 with increasing inhibitor concentrations. The effect on growth and viability was investigated by monitoring the *A*<sub>600</sub> and the number of CFU for 5 h after addition of the antibacterial compound. For CFU quantification culture aliquots were serially diluted in phosphate-buffered saline and plated on Isosensitest agar. After incubation for 18 h at 37 °C visible colonies were counted.

**Determination of the Susceptibility of Chinese Hamster Ovary Cells**—In this assay the fluorescent DNA-specific dye DAPI (Sigma-Aldrich) was used as viability marker. Chinese hamster ovary cells were cultivated in RPMI medium (Invitrogen) containing 9% fetal calf serum, 1.8 mM L-glutamine, and penicillin (100 IU)/streptomycin (100 μg/ml) (Roche). A number of 5 × 10<sup>4</sup> cells/ml was incubated with increasing concentrations of test compound in a volume of 1 ml in 24-well cell culture plates (Costar) for 24 h at 37 °C and 5% CO<sub>2</sub>. After cultivation for an additional 48 h in fresh medium in the absence of inhibitor the culture medium was removed and the cells were stained with 0.2 ml of a DAPI solution (5 μg/ml in distilled water). Finally, cells were transferred into a white microtiter plate (Dynatech Laboratories), and fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm.

Microscopy of DAPI-stained *B. subtilis*—A *B. subtilis* 168 culture in Isosensitest broth was treated at an *A*<sub>600</sub> of 0.1 with increasing inhibitor concentrations. At various time points after addition of the inhibitor culture aliquots of 300 μl were removed and mixed with 11 μl of a DAPI solution (150 μg/ml in distilled water). After staining for 15 min on ice the samples were immobilized on an agar-coated microscope slide and analyzed in a fluorescence microscope (Zeiss) at an excitation wavelength of 360 nm and an emission wavelength of 397 nm. For coating the slides were dipped into a solution of 2% agar in distilled water.

**RESULTS**

**Potent Inhibition of Isolated Bacterial DNA Ligases in Vitro**—Based on purified DNA ligase LigA from *E. coli* we developed an assay to screen for low molecular weight compounds as selective inhibitors of bacterial DNA ligases. The successful ligation reaction was quantified by the amount of AMP released by the enzyme upon joining of two oligonucleotides. The AMP was subsequently converted to ATP by adenylyl kinase and pyruvate kinase, and the resulting ATP was finally visualized by luminescence generated by ATP-depend-
Specific Inhibition of NAD$^+$-dependent DNA Ligase

**Pyridochromanones Inhibit DNA Ligase Competitively with Respect to NAD$^+$**—To elucidate the molecular mechanism by which the pyridochromanones inhibit DNA ligase, we determined their influence on the ligase reaction employing the AMP release assay. In the absence of the inhibitor we determined a $K_m$ of 4 μM for NAD$^+$ and of 0.1 μM for the DNA template, which is in good agreement with previously reported data (18). When in our inhibition studies the amount of NAD$^+$ was increased from 0.5 to 64 μM in the presence of varying concentrations of compound 3 (0–400 μM) and saturating DNA amount (0.67 μM) the kinetics indicated competitive inhibition as visualized in a double-reciprocal plot (Fig. 2, A and B). The linear regression using the apparent $K_m$ value leads to a $K_i$ of about 0.094 μM (Fig. 2C). In contrast, the binding of the DNA under saturating NAD$^+$ concentration (64 μM) in the presence of the inhibitor seems to follow a non-competitive binding mode because $V_{max}$ remains reduced even at high DNA concentrations. These findings strongly suggest that the binding site of the pyridochromanones overlaps at least partially with that of the cofactor NAD$^+$.

Studies with a regulated NAD$^+$ mutant of *B. subtilis* 168 support this interpretation. In the strain AL 811 (*trpC2*, $\Delta$nadD, $\Delta$thrC::xyIR-nadD-spec$^3$) the nadD gene coding for nicotinic acid mononucleotide adenylyltransferase, which is essential for NAD$^+$ biosynthesis (19), was deleted at the original locus, and a chromosomal copy was expressed under the control of the xylose promoter. When AL 811 was grown in LB medium supplemented with 0.25% xylose ensuring full expression of NadD, the MIC for compound 3 was 6 μg/ml, whereas under conditions of promoter catabolite repression in the presence of 0.2% glucose the MIC dropped to 1.5 μg/ml. The observation that the lowered cytoplasmic NAD$^+$ level enhances antibacterial activity supports the notion of competition with the cofactor also on the cellular level.

Several experiments indicate that there is no general interaction of the pyridochromanones with DNA. In a gel shift assay plasmid DNA was incubated with rising inhibitor concentrations followed by analysis of its electrophoretic mobility. The pyridochromanones did not effect the migration behavior of the DNA up to the highest concentration tested (25 μM), whereas the intercalating DAPER dye (20) had an IC$_{50}$ below 5 μM. In addition, the ligase inhibitors were not able to displace ethidium bromide from DNA even at a concentration of 250 μM representing a 50-fold molar excess over ethidium bromide.

Consistent with the result that no DNA interaction was observed, the pyridochromanones did not interfere with the activity of DNA gyrase from *M. luteus*, which we chose as an example of other DNA processing enzymes. The supercoiling activity of DNA gyrase was not affected up to 200 μM, whereas an IC$_{50}$ of 60 m was determined for novobiocin, which was employed as reference inhibitor (21). The fact that bacterial gyrase as well as human ligase are not hampered by the pyridochromanones demonstrates that the basis of the activity of this compound class is the targeted inhibition of NAD$^+$-dependent DNA ligases but not general, nonspecific disturbance of the DNA topology.

**Inhibition of DNA Ligase Is the Basis for Bacterial Cell Death**—The pyridochromanones possess substantial antibacterial activity directed primarily against Gram-positive bacteria. Growth inhibition of *B. subtilis* was already described above. Another example is the important human pathogen *S. aureus* for which MIC values in the low μg/ml range were obtained (Table II). As exemplified for compound 3 the activity was bactericidal with a concentration-dependent reduction in the number of viable bacteria, e.g., a decrease of CFU by 2 log units over 2 h at a concentration of 2 times the MIC (Fig. 3). Intact *E. coli* cells were not affected but they became susceptible when the integrity of the outer membrane was disturbed by the permeabilizing agent polymycin B nonapeptide (22). This result implies that once the outer membrane barrier is overcome the target is inhibited also in *E. coli*. In accordance with the result that no inhibition of human DNA ligase I was detected, the growth of the fungus *C. albicans* was not impaired (Table II). Furthermore, no cytotoxicity was observed for Chinese hamster ovary cells even though the staining procedure chosen to determine cell viability relied on the DNA-specific dye DAPI (23) and was thus especially sensitive to DNA damage (Table II).

A novel antibacterial compound under investigation may prevent the activity of a desired isolated enzyme in a biochemical *in vitro* assay and may also inhibit the growth of intact bacteria. However, it is still conceivable that the two activities are unrelated because the antibacterial effect is based on a rather nonspecific interference with a multitude of essential cellular functions. Such a global disturbance of cell viability may be caused by disruption of membrane integrity, by DNA intercalation, or by elevated chemical reactivity of the compound. To exclude such undesired effects in the case of the pyridochromanones we determined their influence on DNA ligase in the intact bacterial cell. For this purpose we used the *E. coli* DNA ligase mutant GR501 harboring the temperature-sensitive *lig*251 mutation in *LigA* (6). Although this strain grew well at 28 °C the increase in cell mass was strongly delayed at 37 °C (Fig. 4). This growth behavior reflects the ligase activity previously measured in this mutant. Although the mutated ligase was fully functional in cell extracts at 25 °C, no residual activity was detected at 40 °C (6). Heterologous expression of plasmid-encoded human DNA ligase I was described previously to restore growth of GR501 at restrictive temperatures (14) and was reproduced by us (Fig. 4). An equal or even slightly better rescue effect was obtained by expressing *S. aureus* DNA ligase in the *E. coli* mutant (Fig. 4). Together these three strains form a convenient system to determine the
**FIG. 2. Mode of DNA ligase inhibition by the pyridochromanones.** Competitive inhibition with respect to NAD$^+$ (A–C) and non-competitive inhibition with respect to DNA (D). A and B, activity of *E. coli* LigA as measured by the “AMP release assay” in the presence of rising concentrations of compound 3 (12.5–400 nM) and NAD$^+$ (0.5–64 μM). The double-reciprocal plot indicates a competitive binding between NAD$^+$ and the pyridochromanones. C, linear regression of $K_m$ versus inhibitor concentration; the $K_i$ value is marked with an arrow. D, kinetics of ligase activity under varying concentrations of DNA (0.08–2.6 μM) and compound 3 (100–800 nM).

**TABLE II**

Antibacterial activity and effect on growth of eukaryotic cells

| Compound | MIC E. coli GR501 | MIC E. coli GR501 + human ligase | MIC S. aureus 133 | MIC C. albicans ATCC 200498 | CHO cells (IC$_{50}$) |
|----------|------------------|---------------------------------|------------------|--------------------------|----------------------|
|          | (ligAts)         | (ligAts) + S. aureus lig         |                   |                          |                      |
| E. coli  | GR501            |                                 |                  |                          |                      |
| 1        | 1.6              | ND$^a$                          | >100             | 4                        | >60                  |
| 2        | 0.4              | 6                               | >100             | 1                        | >60                  |
| 3        | 0.2              | 6                               | >100             | 1                        | >60                  |
| 4        | 0.4              | ND$^a$                          | >100             | 2                        | >60                  |
| 5        | 0.1              | ND$^a$                          | >100             | 1                        | ND                   |

$^a$ ND, not determined.

**effect of an inhibitor of bacterial ligases in the cellular environment.** Although non-complemented GR501 was highly susceptible for the pyridochromanones at elevated temperatures, the antibacterial activity of this compound class was completely abolished in the presence of the human ligase (Table II). That the bactericidal effect was totally overridden by the expression of the human enzyme proves that inhibition of DNA ligase is indeed the cause for bacterial cell death and confirms again that the function of the human ligase is not impaired. In contrast, *S. aureus* ligase did not rescue the *E. coli* mutant from the activity of the inhibitors (Table II), demonstrating that the enzyme from *S. aureus* is also targeted by the pyridochromanones. The observed decrease in susceptibility of the strain supplemented with *S. aureus* ligase in relation to the non-complemented GR501 (e.g. 0.1 versus 6 μg/ml for compound 3, respectively) could be assigned to the low residual activity of the temperature-sensitive *E. coli* ligase compared with the high copy number of overexpressed *S. aureus* ligase.

Also in whole *B. subtilis* cells we obtained proof that the ligase function is disturbed. Microscopic investigation of a pyridochromanone-treated culture (Fig. 5) showed the formation of filaments in accordance with induction of the SOS cascade and
the resulting inhibition of cell division (24). Staining of the DNA in these cells with the fluorescent dye DAPI revealed massive chromosome degradation, consistent with impaired joining of the Okazaki fragments. The phenotype induced by the pyridochromanones in this DNA staining experiment resembled the one observed in the presence of quinolones such as ciprofloxacin (Fig. 5), known to cause DNA strand breaks and degradation after arresting gyrase on the DNA in a covalent ternary complex (25). Induction of the SOS response by pyridochromanones was confirmed by an experiment with *B. subtilis* YB3001. In this strain the *lacZ* reporter gene is coupled to the recA promoter and integrated into the chromosomal amyE locus (26). When a X-gal-containing agar plate was overlaid with a suspension of this strain, expression of β-galactosidase produced a blue halo from the chromogenic substrate around a drop of a compound 3 solution (data not shown).
growth of *B. subtilis* and lead to DNA degradation within the cell. Therefore, also in this case, the NAD$^+$-dependent ligase is effectively inhibited, and the ATP-dependent variants cannot compensate for this defect. It is also noteworthy that some bacteria have more than one NAD$^+$-dependent DNA ligase. In the *E. coli* genome *ligB* was identified by virtue of its sequence similarity with the primary NAD$^+$-dependent *ligA*, and *ligB* homologues were also detected in *Yersinia* and *Salmonella* (28, 29). For *E. coli* *LigB* the NAD$^+$-dependent nick-joining activity was demonstrated, but the specific activity was only 1% that of *LigA* (28). Thus, the precise function of *LigB* still has to be elucidated; however, *LigB* cannot compensate for the loss of *LigA*.

Different cofactor specificity is a major distinction between eubacterial and eucaytotic DNA ligases and one rationale for why it seems feasible to find specific inhibitors with a preference for one of the two separate ligase families. In our competition studies, the result that the highly specific pyridochromanones bind competitively with respect to NAD$^+$ and not with respect to DNA fits into this picture. The identification of such a type of inhibitor was facilitated by the screening format employed, because the AMP release assay was performed in the range of the *K$_m$* for NAD$^+$ (5 µM) and at a saturating DNA concentration (0.67 µM).

The difference between NAD$^+$ and ATP lies in the presence of the nicotinamide ribose monophosphate moiety whereas the AMP portion occurs in both cofactors. So far, no crystal structure has been published for a eubacterial DNA ligase in a complex with a complete NAD$^+$ molecule, which would show unequivocally the position of the NMN moiety. However, structural information is available for the adenylation domain of the NAD$^+$-dependent ligase from *Bacillus stearothermophilus* (30) and the complete NAD$^+$-dependent enzyme from *Thermus filiformis* with AMP covalently attached (31). With respect to ATP-dependent ligases, crystal structures were obtained for the enzyme from bacteriophage T7 with bound ATP (32) and for the chlorella virus ligase-AMP intermediate (33). These structures show the overall architecture of the different enzymes as well as the location and structure of the AMP-binding pocket. Each of the four DNA ligases have in common that they contain two domains divided by a deep cleft, an N-terminal adenylation domain in which the cofactor-binding site is located and a C-terminal DNA-binding domain. Despite the lack of sequence homology between the two families the AMP-binding pocket shows remarkable structural similarity, and most of the few amino acids that are conserved among the two classes make important contacts with the AMP moiety. In the NAD$^+$-dependent ligase from *T. filiformis* the AMP is located in a pocket between two β-sheets of subdomain 1b and the covalent contact is established to Lys$^{116}$ (31). This pocket is spacious enough for the AMP moiety but is too small for the NMN portion, which is believed to extend along the surface of the adenylation domain. In a recent site-directed mutagenesis study with *E. coli* *LigA* several conserved residues were identified in subdomain 1a, which are crucial for the interaction with the nicotinamide nucleoside. The authors propose that this subdomain, which occurs only in NAD$^+$-dependent ligases, performs a conformational change upon binding of the cofactor that puts NAD$^+$ in the proper orientation for hydrolysis of the phosphoanhydride bond (34).

To obtain a first hint on the molecular-binding mode of the pyridochromanones, we selected for a highly resistant colony (MIC ≥ 60 µg/ml) of *S. aureus* 133 on compound 3-containing agar plates. Sequencing of the *lig* gene revealed a transition of guanine for adenine in position 1117 resulting in an amino acid exchange of Ala$^{373}$ for Thr. This alanine residue is widely conserved among Gram-positive and Gram-negative euabacteria, although a few deviations exist such as exchange for Ser in *Helicobacter pylori* and *Clostridium perfringens* or even Arg in *Borrelia burgdorferi*. The corresponding residues in *B. stearothermophilus* and in *T. filiformis* are Ala$^{375}$ and Ala$^{378}$, respectively, which reside within the so-called oligomer-binding fold, a hinge region that connects the N-terminal and C-terminal domains. In the *T. filiformis* ligase structure Ala$^{375}$ is located directly opposite the entrance of the AMP-binding pocket (Fig. 6). It is well conceivable that an inhibitor which occupies this...
position hampers the access of the cofactor to its binding site with concomitant maintenance of specificity.

In summary, the example of the pyridochromanones clearly demonstrates that by direct interaction with a region which differentiates NAD\(^+\)-dependent and ATP-dependent DNA ligases rather than by mere intercalation-like interaction with the DNA substrate, it is possible not only to selectively target the eubacterial DNA ligases with high potency but also to avoid the interference with the activity of other DNA processing enzymes.

Acknowledgments—We thank Claudia Byl, Andrea Felder, Markus Keil, Andreas Krüger, Heike Neuhaus, Sabine Raschat, Gabriele Richroth, and Ute Sauer (Bayer AG) for expert technical assistance. Lars Keil, Andreas Krüger, Heike Neuhaus, Sabine Raschat, Gabriele Richroth, and Ute Sauer (Bayer AG) for providing the NadD mutant and the topoisomerase \(\text{DNA gyrase and intercalation assay, Frank Bauch (Bayer AG)}\) for determination of the Candida MIC, and Christoph Freiberg and Heiner Appel (Bayer AG) for providing the NadD mutant and the topoisomerase I production strain, respectively. In addition, we thank Ronald E. Yabson (University of Baltimore) for the RecA reporter strain and Deborah Barnes (Clare Hall Laboratories, Herfordshire, UK) for the plasmid pBluescript SK. For DNA ligase genes, we thank Dr. U. Sauer (Bayer AG) for the plasmid pBluescript SK.

REFERENCES

1. WHO/CDSS/CSR/DRS/2001.1. (2001) World Health Organization, Geneva.
2. Lehman, I. R. (1974) Science 186, 790–797.
3. Timson, D. J., Singleton, M. R., and Wigley, D. B. (2001) Mutat. Res. 460, 301–318.
4. Tomkinson, A. E., and Mackey, Z. B. (1998) Mutat. Res. 407, 1–9.
5. Wilkinson, A., Day, J., and Bowater, R. (2001) Mol. Microbiol. 40, 1241–1248.
6. Dermody, J. J., Robinson, G. T., and Sternberg, J. (1979) J. Bacteriol. 139, 701–704.
7. Pettit, M. A., and Ehrlich, S. D. (2000) Nucleic Acids Res. 28, 4632–4648.
8. Kaczmarek, F. S., Zaniecki, R. P., Gootz, T. D., Danley, D. E., Mansour, M. N., Griffier, M., Kamath, A. V., Cronan, M., Mueller, J., Sun, D., Martin, P. K., Benton, B., McDowell, L., Biek, D., and Schmid, M. B. (2001) J. Bacteriol. 183, 3016–3024.
9. Montecucco, A., Fontana, M., Focher, F., Lastingi, M., Spadari, S., and Ciarrocchi, G. (1991) Nucleic Acids Res. 19, 1067–1072.
10. Montecucco, A., Lastingi, M., Rossignol, J. M., Elder, R. H., and Ciarrocchi, G. (1993) Biochem. Pharmacol. 45, 1536–1539.
11. Tan, G. T., Lee, S., Lee, I.-S., Chen, J., Leitner, P., Besterman, J. M., Kinghorn, A. D., and Pezzuto, J. M. (1996) Biochem. J. 314, 995–1000.
12. Ciarrocchi, G., MacPherr, D. G., Deady, L. W., and Tilley, L. (1999) Antimicrob. Agents Chemother. 43, 2766–2772.
13. Suhara, A., Sugihara, H., and Ukawa, K. (January 16, 1992) German patent DE2809720.
14. Kodama, K., Barnes, D. E., and Lindahl, T. (1991) Nucleic Acids Res. 19, 6095–6099.
15. Le Peq, J.-B., and Pacletti, C. (1967) J. Mol. Biol. 27, 87–106.
16. Anagnostopoulos, C., and Spitzizen, J. (1961) J. Bacteriol. 81, 741–746.
17. Soderhall, S., and Lindahl, T. (1973) J. Biol. Chem. 248, 672–675.
18. Modrich, P., and Lehman, I. R. (1974) J. Biol. Chem. 249, 7569–7581.
19. Olland, A. M., Underwood, K. W., Czerwinski, R. M., Lo, M. C., Aulabaugh, A., Bard, J., Shahl, M. L., Somers, W. S., Sullivan, F. X., and Chopra, R. (2002) J. Biol. Chem. 277, 3698–3707.
20. Liu, Z., and Rill, R. L. (1996) Anal. Biochem. 236, 139–145.
21. Sugino, A., Hijikata, N. P., Brown, P. O., Feebless, C. L., and Cozzarelli, N. R. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4838–4842.
22. Vaara, M. (1991) Drugs Exp. Clin. Res. 17, 437–443.
23. Kapuscinski, J. (1995) Biotech. Histochem. 70, 220–233.
24. Love, P. E., and Yabson, R. E. (1961) J. Bacteriol. 160, 910–920.
25. Driess, K., and Zhao, X. (1997) Microbiol. Mol. Biol. Rev. 61, 377–392.
26. Cheo, D. L., Bayles, K. W., and Yabson, R. E. (1992) Biochimie (Paris) 74, 755–762.
27. Cheng, C., and Shuman, S. (1997) Nucleic Acids Res. 25, 1369–1374.
28. Sriskanda, V., and Shuman, S. (2001) Nucleic Acids Res. 29, 4930–4934.
29. Parkhill, J., Wren, B. W., Thomson, N. R., Tishh, K. D., Halfen, M. T., Prentice, M. B., Pentiah, M. J., Reiner, M. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeno-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Kalashnikov, A. V., Leather, S., Meule, S., Oyston, P. C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrett, B. G. (2001) Nature 413, 523–527.
30. Singleton, M. R., Hannasonnson, K., Timson, D. J., and Wigley, D. B. (1999) Structure 7, 35–42.
31. Lee, J. W., Chang, C., Song, H. K., Moon, J., Yang, J. K., Kim, H.-K., Kwon, S.-T., and Sub, S. W. (2000) EMBO J. 19, 1119–1129.
32. Subramanias, A. H., Doherty, A. J., Ashford, S. R., and Wilgey, D. B. (1996) Cell 85, 607–615.
33. Odell, M., Sriskanda, V., Shuman, S., and Nikolov, D. B. (2000) Mol. Cell 6, 1183–1193.
34. Sriskanda, V., and Shuman, S. (2002) J. Biol. Chem. 277, 9695–9700.