CHIMIA 2001, 55, No. 4

Chimia 55 (2001) 291–294

ISSN 0009–4293

Evolutionary Approaches to Study Cytochrome *c* Peroxidase

André Iffland, Petra Tafelmeyer, Susanne Gendreizig, and Kai Johnsson*

Abstract: Directed molecular evolution of enzymes and proteins has emerged as an extremely powerful method to create proteins with novel properties, both for practical applications as well as for mechanistic studies. To demonstrate the underlying principles of this approach, we describe here our work on the heme-containing cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae*. Using directed evolution, we changed the substrate specificity of CCP from the protein cytochrome *c* to a small organic molecule with the best mutants possessing up to 300-fold higher activity against a phenolic substrate. In addition to novel insights into the mechanism of peroxidases, the results illustrate the ability of directed molecular evolution technologies to deliver solutions to biochemical problems that would not be readily predicted by rational design.

Keywords: Directed evolution · Enzymes · Enzyme mechanisms · Peroxidases

The design and generation of enzymes with new activities and specificities is a formidable task, important for our understanding of enzyme mechanisms and for practical applications. In general, there are two complementary approaches to this problem; protein engineering using rational design and the directed evolution of the enzyme of interest. The rational design of new enzyme activities relies on structural information and a detailed understanding of the reaction mechanism. It can be considered as a direct test of our knowledge of enzyme mechanisms and protein folding. Evolutionary approaches, in contrast, rely on challenging a large collection of randomly generated mutants with a particular problem (such as a new substrate) and selecting the mutants with the desired properties [1]. Here, neither structural information nor a detailed understanding of the reaction are a prerequisite.

We have become interested in the question how the protein scaffolds in heme-containing proteins control the chemistry and reactivity of the versatile cofactor. As a starting point into this intriguing problem at the interface of chemistry and biology, we have decided to change the properties of the cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae* using directed evolution [2]. CCP catalyzes the oxidation of ferrocytochrome *c* (Cc^{2+}) by H_2O_2 (Fig. 1, Scheme 1) [3]. It is a monomeric protein of 294 residues and a member of the superfamily of plant, fungal and bacterial peroxidases. The substrate specificities within this superfamily of peroxidases vary considerably with substrates ranging from a protein, such as cytochrome *c* in the case of CCP, to classical peroxidase substrates, such as phenols and anilines in case of horseradish peroxidase (HRP) [4]. In addition to its natural substrate, CCP can oxidize small organic and inorganic substrates as well, although its activity is orders of magnitude below that of HRP. In CCP, Cc^{2+} and small organic substrates are oxidized at different sites of the peroxidase (Fig. 2). As in HRP, small substrates approach the heme of CCP from its distal side and the electron transfer is believed to take place near the δ-meso edge of the porphyrin [5]. Within the complex formed between Cc^{2+} and CCP, the two hemes are more than 17 Å apart and the electron transfer is believed to occur *via* the proximal side of the heme of CCP [6]. We reasoned that changing the substrate specificity of CCP towards a small molecule, such as guaiacol, using directed evolution should help us to identify residues that play a decisive role in controlling the reactivity and specificity of this peroxidase.

Critical for the directed molecular evolution of an enzyme is the design of a selection scheme which allows the mutant with the desired activity to be isolated from a large population of inactive or less active mutants. In the case of peroxidases we can use the fact that various peroxidase substrates form colored products or by-products (Scheme 2). For example, the initial product of the oxidation of guaiacol, the corresponding phenoxy radical, spontaneously dimerizes to the intensely brown tetraguaiacol (Scheme 2A) [7]. Taking advantage of this and the fact that CCP can be functionally expressed in *E. coli*, we have developed a simple assay that relies on the visual inspection of CCP-expressing *E. coli* colonies after exposure to the substrate and H_2O_2. The fastest staining colonies are amplified and the plasmid carrying the CCP gene isolated. Substrates that can be used in this assay include phenols, aromatic amines, classical peroxidase substrates, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and luminol (detection based on chemiluminescence). About 5000 different colonies can be screened on a single plate.

The next important step is to create variation in the gene of interest. For the creation of CCP libraries, we amplified the CCP gene under conditions where the error rate for DNA replication by DNA

*Correspondence: Prof. Dr. K. Johnsson
Institut de Chimie Organique
Université de Lausanne
CH-1015 Lausanne
Tel.: +41 21 692 39 56
Fax: +41 21 692 39 65
E-Mail: kai.johnsson@ico.unil.ch

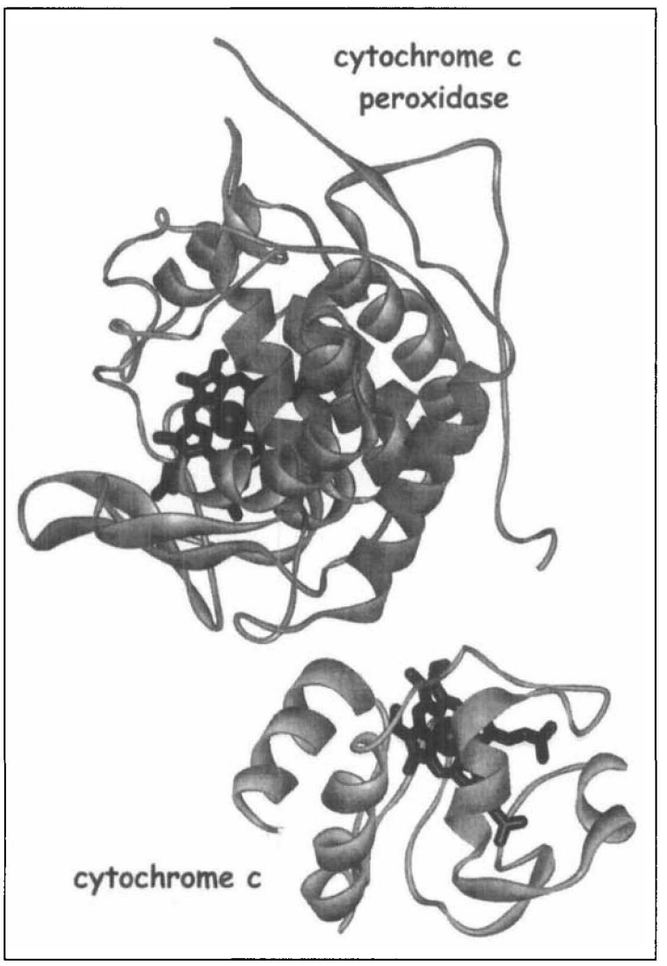


Fig. 1. Crystal structure of the complex of CCP with its natural substrate Cc^{2+}. Shown are the two protein backbones and the two heme molecules [6].

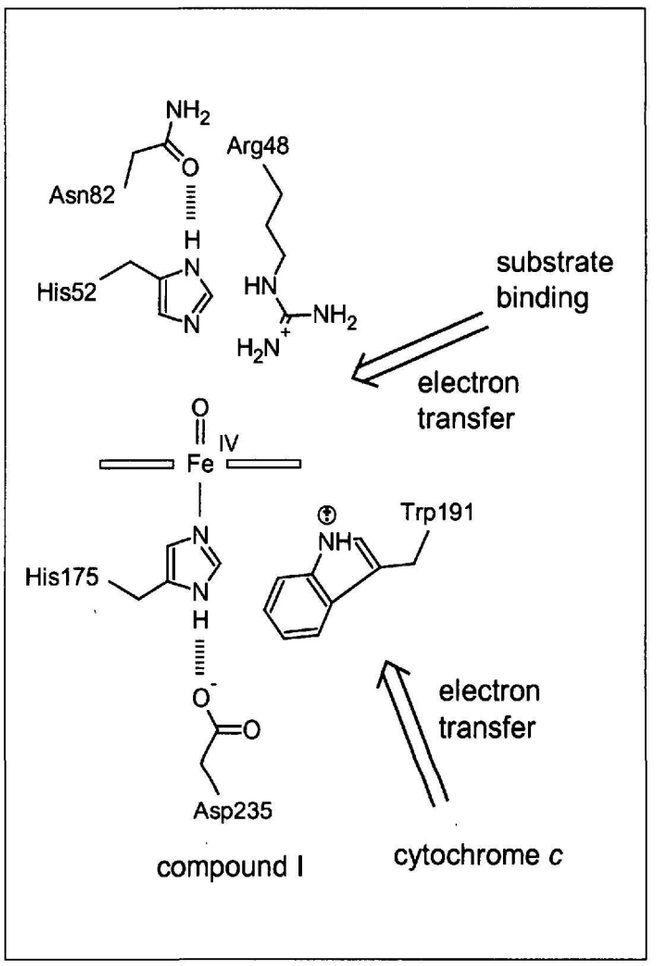
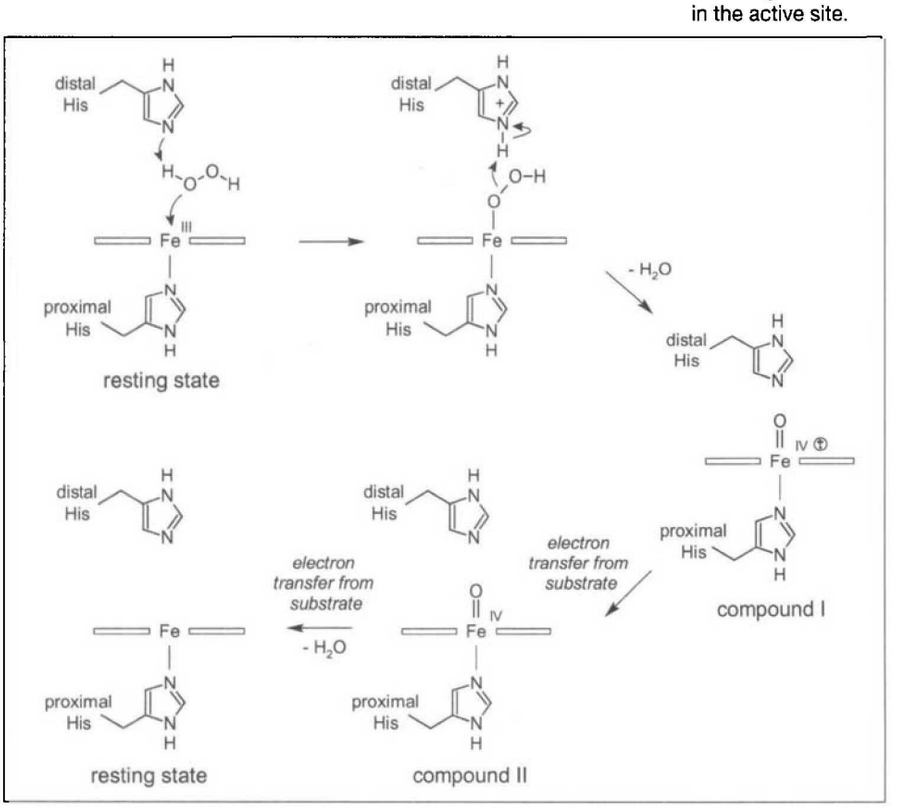


Fig. 2. Schematic drawing of the structure of compound I of CCP and location of the different binding sites for the natural substrate Cc^{2+} and small organic molecules. Also shown are a few of the critical residues in the active site.



Scheme 1. General reaction mechanism for peroxidases. For clarity, only a schematic drawing of the heme and the distal and the proximal histidines are shown. The cation radical of compound I might be either an indolyl cation radical as in CCP or a porphyrin π cation radical as in HRP. In the case of CCP, the substrate donating one electron to compound I and one electron to compound II is ferrocytochrome c (Cc^{2+}), which is oxidized to ferricytochrome c (Cc^{3+}). Two molecules of Cc^{2+} are oxidized per molecule of H_2O_2. In the case of HRP, possible substrates are phenols or anilines.

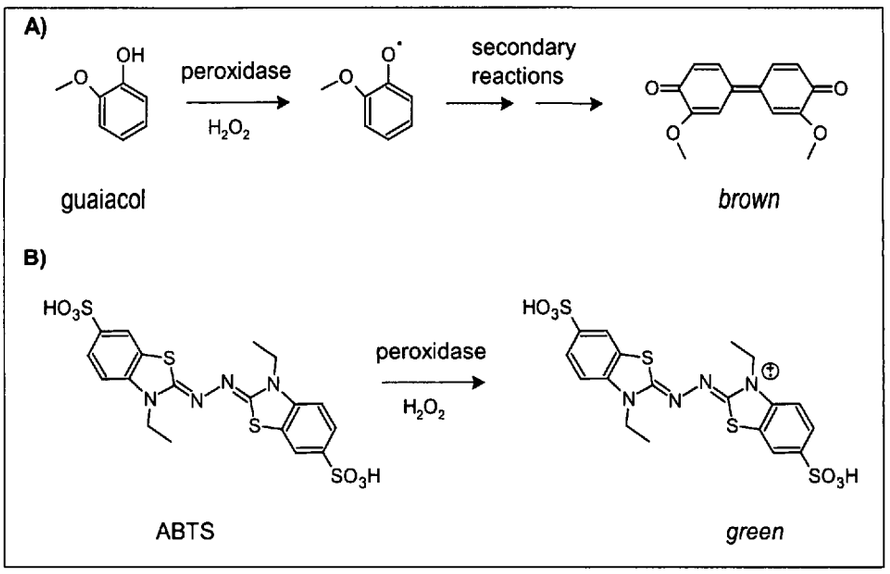
CHIMIA 2001, 55, No. 4

polymerases is increased (*i.e. error prone* PCR). This leads to the incorporation of random point mutations throughout the whole CCP gene. The error rate for these low-fidelity DNA amplifications is, depending on the conditions used, about 0.2% [8]. Thus, most members of the library possess about two mutations in their CCP genes and, consequently, one to two amino acid mutations. Much higher mutation rates increase the chance of incorporating disruptive amino acid mutations, leading to libraries mostly comprised of inactive clones. On the DNA level, there are about 3000 possible single mutants and 3.6×10^6 possible double mutants. Considering that about 5000 CCP clones can be screened in a single experiment, the complete screening of all possible double mutants is, for practical purposes, impossible. As the majority of the mutations at the amino acid level require more than one base change per codon (for example mutating Gly (GGC) to Pro (CCC)), the chances of observing these mutations when only 10^3–10^4 mutants are screened are extremely small. It is important to keep these limitations in mind when planning and interpreting a directed evolution experiment.

The improvements in activity brought about by single point mutations will not be very dramatic in most cases. If we assume that beneficial mutations are additive, we could, of course, repeat the cycle of random mutagenesis and screening many times. However, there is an extremely elegant technique, DNA shuffling, to speed up this process [9]. In this technique, the genes of the most active mutants are amplified, randomly fragmented and subsequently reassembled using PCR, allowing for recombination between different mutants. Libraries prepared in this way are recombinant progeny of the previously selected mutants and can then be used in subsequent selections. This process of 'molecular breeding' can be considered as one of the most important technical advances in the field of directed evolution.

In the case of the directed molecular evolution of CCP, we performed three rounds of random mutagenesis and DNA shuffling, selecting the fastest staining colonies in each round [2]. After the third round, colonies started to stain after 5 min of exposure to guaiacol and H_2O_2, whereas colonies expressing wild-type CCP did not stain within 60 min.

Eleven clones obtained after the third round were characterized by sequencing and their activity measured. On average, each clone has about five point mutations, the lowest number being four and the highest eight. The activity of the most active clone with guaiacol increased by a factor of 300, whereas the substrate specificity for guaiacol relative to the natural substrate increased up to 1000-fold. In general, mutations leading to faster staining of colonies can be classified in three groups: those that increase the specific activity against guaiacol, those that increase the reactivity of the peroxidase in general, and those that increase the concentration of the holo-form of the enzyme inside *E. coli* [2]. Mutations that were repeatedly found in independent clones are distributed over the whole structure of the enzyme (Fig. 3). A mutation that was found in all selected clones and which alone increases the activity of CCP towards guaiacol by a factor of 70 is Arg48 to His. This result is striking since Arg48 is one of only nine amino acids that are completely conserved in the superfamily of plant, fungal and bacterial peroxidases, including those that possess a high activity with guaiacol. Arg48 is located on the distal side of the heme next to the equally conserved distal histidine and has been shown to be important for the stabilization of compound I (Fig. 2, 3). How can the dramatic and specific increase of the activity with guaiacol due to the mutation Arg48His be rationalized? The oxidation of phenols by HRP has been a topic of intense research and a scheme of the mechanism is shown in Scheme 3 [10]. In short, electron transfer from the substrate to the heme is accompanied by simultaneous proton transfer from the phenol either to the distal histidine (in the case of compound I) or the ferryl oxygen (in the case of compound II). A possible explanation therefore would be that mutating Arg48 to His in CCP might increase the steric access of guaiacol to the ferryl oxygen or a general base (presumably His52) in the distal cavity, facilitating simultaneous proton and electron transfer. Alternatively, His48 itself might function as a general base catalyst. The latter explanation would suggest that only an amino acid capable of general acid-base catalysis at this position would lead to an increase in activity towards phenols. To discriminate between the two possibilities, we introduced a synthetic oligonucleotide into the gene of the wild type CCP, where the codon for Arg48 had been randomized [11], an approach which is known as saturation mutagenesis. The resulting library thus contains those amino acids at position 48 that are not accessible by mutating only one of the three bases in the codon of Arg48. Screening this small library of 20 mutants against guaiacol as a substrate allowed us to identify four mutations at position Arg48 that led to significantly higher activity with this substrate than wild type: Arg48Gln, Arg48His, Arg48Thr and Arg48Ile. The mutant Arg48Gln had the highest activity with guaiacol, being about 300-fold more active than wild type. Consequently, our results indicate that Arg48 in CCP is not only important in stabilizing compound I, but it also plays the role of gatekeeper in the distal cavity, controlling the access of molecules to the ferryl oxygen as well as to the distal base.



Scheme 2. Structure of two of the substrates used in the directed evolution of CCP and the products of their oxidation. In both cases, the intense color of the product allows the identification of *E. coli* colonies expressing CCP mutants with (increased) activity against the substrate.

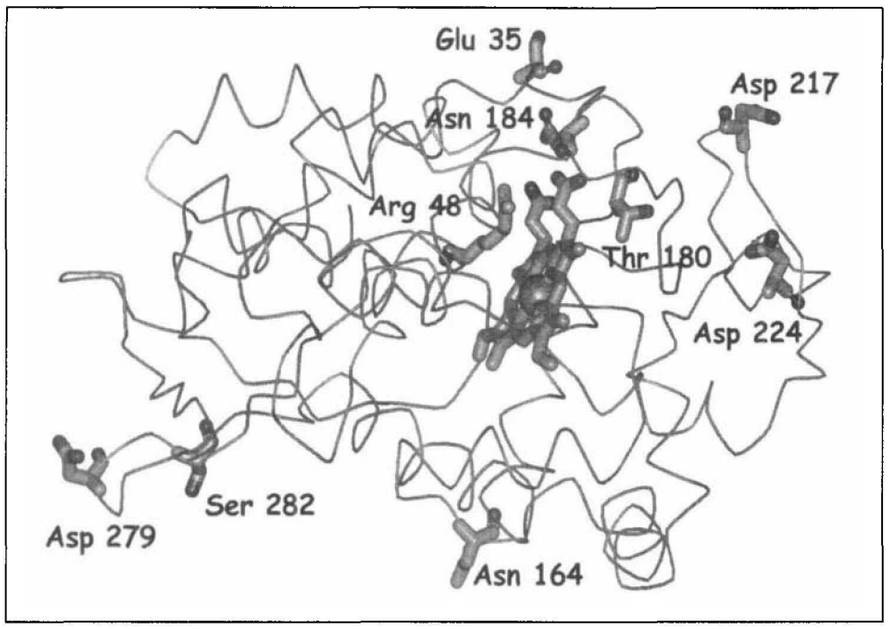
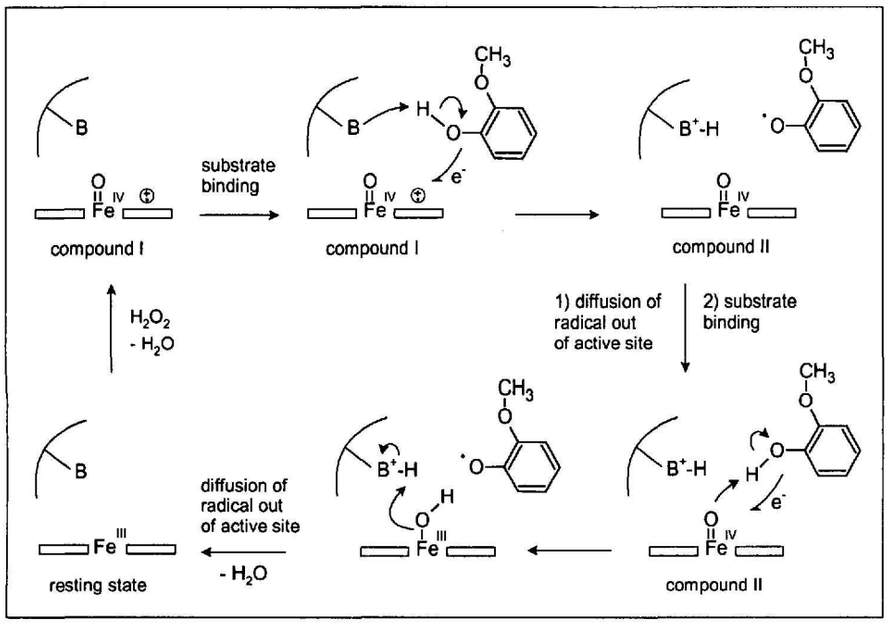


Fig. 3. Residues in the structure of wild type CCP that have been mutated in the course of the directed evolution towards an increased activity against guaiacol and ABTS. Only the mutations that have been found in more than one clone are shown. Asp217 and Asp224 are the residues that have been mutated in the selections with ABTS.



Scheme 3. Proposed mechanism for the oxidation of phenols by HRP [11]. B represents the distal histidine of HRP.

One question arising from these experiments was whether we would find the same mutations in CCP when using a different substrate in the screening assay. We therefore selected for CCP mutants with increased activity toward the classical peroxidase substrate 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Scheme 2B). Using a combination of *error-prone* PCR, DNA shuffling and saturation mutagenesis, we were able to identify mutants that possessed an activity against ABTS up to 30-fold higher [12]. Surprisingly, the responsible mutations, Asp217Pro and Asp224Tyr, reside on the surface of the protein and are not in the vicinity of the distal cavity (Fig. 3). One possible explanation is that the mutations create a binding site for ABTS on the surface of CCP and that the electron transfer from the substrate, as in the oxidation of cytochrome c by CCP, occurs *via* the protein scaffold. Two independent findings support this tempting speculation. First, Asp224 has been suggested to be part of a second binding site of CCP for Cc^{2+}, which shows high activity in Cc^{2+} oxidation, indicating that efficient electron transfer at this site is possible [13]. In addition, there have been recent reports on other peroxidases of this superfamily, which bind and oxidize their substrate not in the distal cavity of the protein, but in different areas on the surface of the protein [14][15].

In summary, our experiments on the directed molecular evolution of CCP have shown that this is a powerful method to rapidly generate CCP mutants with interesting properties, leading to an increase in our understanding of the structure–function relationship of this enzyme. The unpredictability of the results obtained in these experiments demonstrates both the power of evolutionary approaches in general as well as our still limited understanding of the chemistry of heme-containing enzymes.

Acknowledgements

Funding of this work was provided by the Swiss National Science Foundation, the Volkswagen-Stiftung and the Bundesamt für Bildung und Wissenschaft.

Received: February 8, 2001

[1] a) F.H. Arnold, A.A. Volkov, *Curr. Opin. Chem. Biol.* **1999**, *3*, 54–59; b) for a review on the directed evolution of heme-containing enzymes see J.R. Cherry, *Curr. Opin. Biotechnol.* **2000**, *11*, 250.
[2] A. Iffland, P. Tafelmeyer, C. Saudan, K. Johnsson, *Biochemistry* **2000**, *39*, 10790.
[3] H.R. Bosshard, H. Anni, T. Yonetani, in 'Peroxidases in chemistry and biology', Ed. I. Everse, K.E. Everse, M.B. Grisham, CRC press, Boca Raton, **1991**, vol. 2, p. 52–78.
[4] K.G. Welinder, *Curr. Opin. Struct. Biol.* **1992**, *2*, 388.
[5] G.D. DePillis, B.P. Sishta, A.G. Mauk, P.R. Ortiz de Montellano, *J. Biol. Chem.* **1991**, *266*, 19334.
[6] H. Pelletier, J. Kraut, *Science* **1992**, *258*, 1748.
[7] D.R. Doerge, R.L. Divi, M.I. Churchwell, *Anal. Biochem.* **1997**, *250*, 10.
[8] H. Zhao, L. Giver, Z. Shao, J.A. Affholter, F.H. Arnold, *Nat. Biotechnol.* **1998**, *16*, 258.
[9] W.P. Stemmer *Nature* **1994**, *370*, 389.
[10] A. Henriksen, A.T. Smith, M. Gajhede, *J. Biol. Chem.* **1999**, *274*, 35005.
[11] A. Iffland, K. Johnsson, manuscript in preparation.
[12] A. Iffland, S. Gendreizig, P. Tafelmeyer, K. Johnsson, manuscript submitted.
[13] V.W. Leesch, J. Bujons, A.G. Mauk, B.M. Hoffman, *Biochemistry* **2000**, *39*, 10132.
[14] W.A. Dyle, W. Blodig, N.C. Veitch, K. Piontek, A.T. Smith, *Biochemistry* **1998**, *37*, 15097.
[15] E.H. Bursey, T.L. Poulos, *Biochemistry* **2000**, *39*, 7374.