Altering Glucose Oxidase to Oxidize D-Galactose through Crosslinking of Imprinted Protein

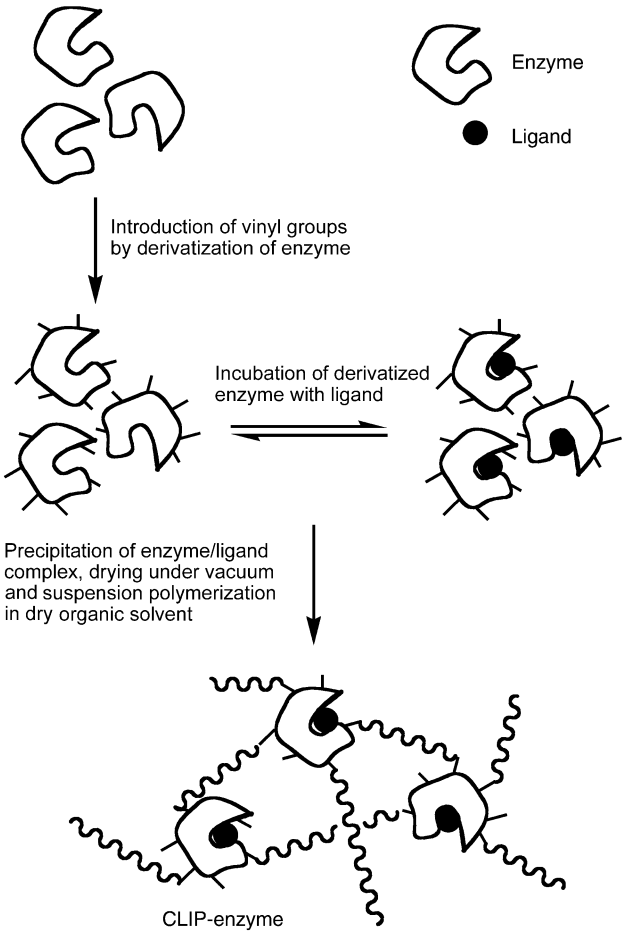
Alankar Vaidya,[a] Alexander Borck,[b] Anja Manns,[a] and Lutz Fischer*[a]

Dedicated to Dr. Matthias Gabler in memoriam.

In the past "molecular imprinting" was mainly confined to the separation of organic molecules and to nonenzymatic catalysis.[1–3] With advancements in this technology, its potential and utility in the field of biotechnology was also realized.[4] In order to improve its applicability, new variations in the conventional imprinting technique have emerged, such as "bioimprinting",[5, 6] metal chelation imprinting,[7] affinity imprinting,[8, 9] and a combination of immobilization and "bioimprinting".[10, 11] The memory effect of enzymes caused by bioimprinting without a further immobilization step was found to depend on the water content of the medium and was completely lost when the reaction was carried out in the presence of a certain amount of water.[5, 12, 13] This was exemplified by the fact that, in aqueous medium, renaturation of a bioimprinted protein to its original native conformation resulted in the loss of imprinted memory.[5, 13] However, water is an indispensable milieu for most enzymatic reactions. Therefore, our research is focused on how to maintain the "bioimprinted memory" of an enzyme not only in organic solvents but also in aqueous solution systems. Here we demonstrate that the combinatorial *cross*linked *imp*rinting approach (we termed it CLIP) overcomes this problem. The CLIP methodology is shown schematically in Scheme 1. Keyes et al. employed a different kind of methodology to alter the catalytic properties of enzymes.[14] In their strategy, the enzyme complexed with ligands was crosslinked by using glutaraldehyde. This whole process was carried out in aqueous buffer, probably because the present imprinting concept in organic solvents was not known to that time.

In our initial attempts[10] monomeric, low-molecular-weight (26 – 28 kDa), nonglycosylated and cofactor-independent proteolytic enzymes, such as chymotrypsin and subtilisin, were selected to demonstrate the feasibility of the CLIP approach to rationally modify their catalytic properties. These proteases, when "bioimprinted" with *N*-acetyl-D-tryptophan, can accept both D- and L-configured substrates, whereas the native enzyme only recognizes the L-form for synthesis of its ethyl ester in dry cyclohexane.[5, 10] Hydrolysis of the D-ester in aqueous phosphate buffer showed that only the crosslinked imprinted enzyme was able to hydrolyze the D-configured substrate; moreover, this reaction took place $10^4 - 10^5$ times faster than the uncatalyzed process.[10] In another example, we reported crosslinked imprinting of a membrane-associated epoxide hydrolase using its substrates (*S*)- or (*R*)-1,2-epoxyoctane as imprint molecules. The resulting CLIP-epoxide hydrolase preparations exhibited enantioselective preference for hydrolysis of either (*S*)- or (*R*)-1,2-epoxyoctane (*ee* = 1.8 and 5.3, respectively) in phosphate buffer.[11] The native nonimprinted epoxide hydrolase was weakly (*R*) selective (*ee*).

In both examples the CLIP technique was applied to introduce a rationally modified enantiorecognition into enzymes for their usage in aqueous buffer systems. Here, glucose oxidase (GO; EC 1.1.3.4; CAS 9001-37-0) was selected as an intricate model



Scheme 1. *Schematic illustration of combinatorial crosslinked imprinting methodology (CLIP). The enzyme of interest is first derivatized and then complexed by using ligands such as substrate analogues or inhibitors in aqueous medium. In the next step, imprinted memory is created by precipitation of protein and drying under vacuum. Subsequently, this imprinted memory is covalently "frozen" by crosslinking the precipitated protein in dry organic solvent. The resulting CLIP enzyme is washed to remove the ligand. It can then be used either in aqueous medium or organic solvent. In the present case of glucose oxidase (GO), the ligand was its competitive inhibitor D-galactose, and the novel catalytic property in aqueous medium was acceptance of D-galactose as a substrate to give D-galactono-1,4-lactone as a product.*

[a] *Dr. A. Vaidya, Dipl.-Ing. A. Manns, Prof. Dr. L. Fischer*
Department of Biotechnology, Institute of Food Technology
University of Hohenheim
Emil-Wolff-Strasse 14, 70599 Stuttgart (Germany)
Fax: (+49) 711-459-4267
E-mail: lfischer@uni-hohenheim.de

[b] *Dr. A. Borck*
Department of Macromolecular Chemistry
Technical University of Braunschweig
Hans-Sommer-Strasse 10, 38106 Braunschweig (Germany)

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enzyme to modify its substrate selectivity and thereby demonstrate the feasibility of CLIP in inducing new catalytic properties in a structurally demanding enzyme that is dimeric, large (160 kDa), glycosylated, and cofactor dependent (two FAD molecules per molecule of enzyme). This is the first report in which the substrate selectivity of GO is broaden through the CLIP strategy on the mature-protein level, which can only be achieved by chemical means, and the resulting modified biocatalyst demonstrates its utility in aqueous medium.

The first step of the CLIP technique is to introduce polymerizable vinyl groups into glucose oxidase (Scheme 1). The reaction of itaconic anhydride with mainly the primary amino groups of proteins is well documented.[15, 16] Itaconic anhydride forms covalent bonds with the NH_2, SH, and OH groups of lysine, cysteine, or tyrosine side chains, which are stable from pH 1 – 12 at temperatures up to 70 °C.[16] It has been reported that 30 lysine, 6 cysteine and 36 tyrosine residues per mole were present in the GO from *Aspergillus niger* used.[17] In our experiments, the GO/anhydride ratio was varied between 1:1 to 1:10 (*w/w*) in order to obtain a degree of protein derivatization in the range of 20 to 100 % (the derivatization degree was estimated by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay;[16] data not shown). It was observed that 70 % acylation (of derivatizable amino acids) was optimum considering the activity of resulting derivatized GO. The 70 %-derivatized GO demonstrated an increased specific activity of 9200 nkat per mg protein relative to native GO (see Table 1).

Table 1. *Kinetic parameters of different glucose oxidase (GO) preparations.*[a]

GO Preparation	Substrate Glucose		Substrate Galactose	
	K_m [mM]	V_{max} [nkat per mg protein]	K_m [mM]	V_{max} [nkat per mg protein]
Native (as reported)[18]	22	2500	n.s.	n.s.
No1 Native GO	22	8500	n.s.	n.s.
No2 Derivatized (70 %) GO	12	9200	n.s.	n.s.
No3 Immobilized GO	19	2400	n.s.	n.s.
No4 CLIP-GO	15	2500	8	1000

[a] Results were average from three independent experiments (deviation ± 10 %). n.s. = not substrate.

Derivatized GO was imprinted with D-galactose, a competitive inhibitor of GO ($K_i \approx 35$ mM) in phosphate buffer, and the GO – galactose complex was precipitated by using *n*-propanol. The resulting protein pellet was dried under high vacuum ($\sim 10^{-5}$ mbar). Our earlier experiences in crosslinked imprinting of enzymes implied that to stabilize the imprinted conformation in aqueous solution it is necessary to use a large amount of crosslinking agent.[10, 11] Hence, in the final step of immobilization, an excess of ethylene glycol dimethacrylate (EGDMA) was used as crosslinker. The crosslinking step was done by UV initiation in dry cyclohexane, a porogenic solvent.

The kinetic parameters K_m and V_{max} for native (No 1), derivatized (No 2), immobilized (No 3), and CLIP (No 4) glucose oxidase (GO) were estimated by using Hanes plot. The corresponding data are shown in Table 1. The K_m value of native GO was found to be the same as reported earlier by Scott, although V_{max} of our GO preparation was significantly higher.[18] If GO was acylated, the K_m decreased from 22 to 12 mM, and the V_{max} value increased from 8500 to 9200 nkat per mg protein, as mentioned above. After crosslinking of derivatized GO, the V_{max} (2400 nkat per mg protein) was about 28 % of the value obtained with native GO. In comparison, α-chymotrypsin immobilized by the same method demonstrated only about 12 % V_{max} of the free native enzyme during hydrolysis of *N*-acetyl-L-tryptophan ethyl ester,[10] and immobilized epoxide hydrolase of *Rhodotorula glutinis* resulted in about 30 – 60 % of initial activity relative to the free enzyme.[11]

The most remarkable feature of CLIP-GO was the acceptance of galactose as a novel substrate ($K_m = 8$ mM). CLIP-GO exclusively catalysed the oxidative conversion of galactose to galactono-1,4-lactone (the product was analyzed by HPCE, HPLC, ^1H and ^{13}C NMR spectroscopy). It was confirmed that the spectral data were in accordance with the proposed structure of the product published by El Khadem et al.[19]

A separate control experiment was conducted in which galactose was converted by native galactose oxidase (EC 1.1.3.9; CAS 9028-79-9), the well-known oxidase for the enzymatic conversion of galactose. The product of galactose oxidase was identified as D-galactonohexodialdose, as expected and previously published.[20] To gain further proof of the different regioselectivity, methyl-α- and methyl-β-D-galactopyranoside were incubated with either native galactose oxidase or CLIP-GO. Only native galactose oxidase was able to convert these C1-protected monosaccharides (84 and 78 % activity, respectively, relative to D-galactopyranoside), whereas CLIP-GO could not oxidize these C1-methylated D-galactopyranosides at all. Thus, all these results indicate that the oxidative catalytic conversion of galactose to galactono-1,4-lactone by using CLIP-GO follows the intrinsic C1-regioselectivity of the enzyme and leads to a different product than with the C6-regioselective galactose oxidase.

The enzymatic conversions of glucose or galactose by GO preparations No 1, 3, and 4 were scaled up to 50 mL batches (the protein concentration was kept constant at 50 μg mL^{-1}, corresponding to 20 mg polymer per mL in case of immobilized or CLIP-GO). The specific conversion rates (mM product per mg protein per h) with native, immobilized and CLIP-GO are shown in Table 2. The highest conversion rate for glucose (14.1 mM glucono-1,5-lactone per mg protein per h) was found for the native enzyme. The conversion rates for glucose with immobilized and CLIP-GO were lower and similar to each other (ca. 8 mM product per mg protein per h). The immobilized GO and CLIP-GO reached approximately 90 % of the extrapolated theoretical yield, whereas native GO resulted in only about 46 %. This is explained by the stability effect due to covalent crosslinking in case of immobilized GO and CLIP-GO. Furthermore, the CLIP-GO lost only 10 % of its initial activity on continuous reuse for six cycles (data not shown).

The major important finding of the present investigation is the exclusive oxidation of galactose to galactono-1,4-lactone by CLIP-GO with surprisingly high 42 % of the conversion rate (3.4 mM product per mg protein per h) compared with its natural substrate glucose (8.1 mM product per mg protein per h). With

Table 2. Production of glucono-1,5-lactone and galactono-1,4-lactone by using different glucose oxidase (GO) preparations (1 h, 50 mL scale).[a]

GO Preparation	Yield of Glucono-1,5-lactone [mM per mg protein]		Yield of Galactono-1,4-lactone [mM per mg protein]	
	Experimental	Theoretical[b]	Experimental	Theoretical[b]
No 1 Native GO	14.1	30.6	0	0
No 3 Immobilized GO	8.0	8.6	0	0
No 4 CLIP-GO	8.1	9.0	3.4	3.6

[a] Results were average from three independent experiments (deviation ± 10%). [b] Corresponding to the initial GO preparation activity at the beginning (t_0; see V_{max} in Table 1).

CLIP-GO about 94% of the theoretical yield of galactono-1,4-lactone was produced. The mechanism of this unique acceptance of galactose by CLIP-GO and obvious differences in active-site conformation of the CLIP-GO and the nonimprinted GO preparations on protein structure level are yet not understood. The cross reactivity of CLIP-GO with other sugars will be the subject of more detailed studies in the future. However, the general principle of the CLIP technique, using ligands that bind to the active site of an enzyme, seems to allow altering enzyme properties such as substrate- or enantioselectivity. Limitations might be observed if the enzyme is sensitive to the acylating agent necessary for covalent immobilization or to propanol precipitation and organic solvents. These points have to be investigated empirically.

In summary, GO was acylated by itaconic anhydride, and the resulting derivatized enzyme was bioimprinted with galactose (competitive inhibitor). The bioimprinted memory was covalently stabilized by crosslinking in water-free organic solvent by using excess EGDMA. The most interesting and unique result obtained in the present case was that galactose was accepted as a novel substrate ($K_m = 8$ mM) and that it was oxidatively converted to galactono-1,4-lactone. This new "bioimprinted" catalytic property was not inherent to native GO. To our knowledge, this is the first report in which the substrate spectrum of GO was not only broadened but yielded a new product by directed biochemical modification of the enzyme on a mature-protein level.

Experimental Section

Materials: Glucose oxidase (specific activity 8500 nkat per mg protein) from recombinant *Aspergillus niger*, peroxidase from horse radish (specific activity 225 units per mg protein, one unit activity corresponds to production of 1 mg purpurogallin from pyrogallol in 20 s at pH 6 and 25 °C) were procured from Roche, Mannheim, Germany. Glucose monohydrate, galactose, galactono-1,4-lactone, glucono-1,5-lactone, cyclohexane, *n*-propanol, hydrogen peroxide (30%, *v/v*) were purchased from Fluka, Buchs, Germany. *o*-Dianisidine dihydrochloride and TNBS were obtained from Sigma Chemical Company, Steinheim, Germany. Ethylene glycol dimethacrylate (EGDMA) was purchased from Aldrich, Steinheim, Germany. 2,2′-Azobis(2-methylpropionitrile) (AIBN) and itaconic anhydride were obtained from Acros Organics, New Jersey, USA. All chemicals were of analytical grade and were used as received, except cyclohexane was dried by refluxing over metallic sodium for 12 h and then stored over molecular sieves.

Derivatization of glucose oxidase: The acylation of GO (6 mg mL^{-1}) by using various amounts of itaconic anhydride (6 to 60 mg mL^{-1}) in potassium phosphate buffer (10 mL, 50 mM, pH 6.0; hereafter "working buffer") was carried out by following our earlier reported procedure.[11] To yield 70% derivatization degree, GO and itaconic anhydride were used in the ratio of 1:7 (*w/w*).

Imprinting of GO: A typical procedure for imprinting was as follows. Dry derivatized enzyme (30 mg mL^{-1}) and galactose (54 mg mL^{-1}) were dissolved in potassium phosphate buffer (1 mL, 10 mM, pH 5.0). The mixture was incubated at 25 °C for 30 min. The GO – galactose complex was precipitated by adding *n*-propanol (4 mL, − 20 °C), then it was kept on ice for 10 min. The precipitate was collected by centrifugation at 11 000 rpm for 15 min at 4 °C. The pellet was washed with *n*-propanol (1 mL, − 20 °C) and then dried with a molecular vacuum pump (Alcatel, Drytel 31) for 12 h and kept under the same vacuum till further use.

Crosslinking of derivatized imprinted enzyme: Imprinted derivatized GO (10 mg mL^{-1}) was suspended in dry cyclohexane (1 mL) by using an ultrasonication bath (Branson 2200). AIBN (4 mg) and EGDMA (200 μL) were dissolved in this suspension. The radical polymerization was initiated under UV irradiation ($\lambda = 335$ nm) at 25 °C and was continued for 5 h. The resulting polymer was kept in a refrigerator at 5 °C for 12 h. First the polymer was washed with cyclohexane (2 mL) to remove unreacted crosslinker, then with working buffer (3 × 10 mL). The protein and enzyme activity were checked during aqueous washings; no enzyme leakage was found. The polymer was dried with a molecular vacuum pump. Similarly, a control polymer with nonimprinted derivatized enzyme was also crosslinked (immobilized enzyme).

Bioconversion of glucose or galactose: In an eppendorf tube (2 mL capacity) or a stirred glass reactor (200 mL capacity) with aeration, either glucose or galactose (200 mM) was dissolved in working buffer (1 mL or 50 mL, respectively; pH 6.0), and free enzyme (50 μg mL^{-1}) or polymer-bound enzyme (ca. 20 mg polymer per mL), comprising the same amount of protein, was added. The reaction mixtures (air saturated) were stirred at 1000 rpm under continuous temperature control at 25 °C. Then the reaction was terminated by a heat step (70 °C). The reaction mixture was centrifuged at 13 000 rpm for 10 min, and the supernatant was analyzed by HPCE, HPLC, ^{1}H, or ^{13}C NMR (for details see the Supporting Information).

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