Origins of Life and Evolution of Biospheres (2005) 35: 369–382

THE STRUCTURE AND SYNTHETIC CAPABILITIES OF A CATALYTIC PEPTIDE FORMED BY SUBSTRATE-DIRECTED MECHANISM – IMPLICATIONS TO PREBIOTIC CATALYSIS

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(Received 30 May 2004; accepted in revised form 20 August 2004)

Abstract. Previously, we have shown that a small substrate may serve as a template in the formation of a specific catalytic peptide, a phenomenon which might have had a major role in prebiotic synthesis of peptide catalysts. This was demonstrated experimentally by the formation of a catalytic metallo-dipeptide, Cys_2-Fe^{2+}, around o-nitrophenyl β-D-galactopyranoside (ONPG), by dicyandiamide (DCDA)-assisted condensation under aqueous conditions. This dipeptide was capable of hydrolyzing ONPG at a specific activity lower only 1000 fold than that of β galactosidase. In the present paper we use molecular modeling techniques to elucidate the structure of this catalyst and its complex with the substrate and propose a putative mechanism for the catalyst formation and its mode of action as a "mini enzyme". This model suggests that interaction of Fe^{2+} ion with ONPG oxygens and with two cysteine SH groups promotes the specific formation of the Cys_2-Fe^{2+} catalyst. Similarly, the interaction of the catalyst with ONPG is mediated by its Fe^{2+} with the substrate oxygens, leading to its hydrolysis. In addition, immobilized forms of the catalyst were synthesized on two carriers – Eupergit C and amino glass beads. These preparations were capable of catalyzing the formation of ONPG from β-D-galactose and o-nitrophenol (ONP) under anhydrous conditions. The ability of the catalyst to synthesize the substrate that mediates its own formation creates an autocatalytic cycle where ONPG catalyzes the formation of a catalyst which, in turn, catalyzes ONPG formation. Such autocatalytic cycle can only operate by switching between high and low water activity conditions, such as in tidal pools cycling between wet and dry environments. Implications of the substrate-dependent formation of catalytically active peptides to prebiotic processes are discussed.

Keywords: anhydrous synthesis, catalytic peptides, iron, prebiotic catalysis

1. Introduction

In a previous paper, we showed experimentally that an assembly of free amino acids can mimic the reactions of carbonic anhydrase, catalase and β-galactosidase (Bar-Nun *et al.*, 1994). In the latter case, cysteine was found to be the most active amino acid in catalyzing the hydrolysis of o-nitrophenyl β-D-galactopyranoside (ONPG) into o-nitrophenol (ONP) and β-D-galactose. Further, we showed

experimentally (Kochavi *et al.*, 1997) the formation of a catalytically-active dipeptide, which, was identified as $\overset{Cys}{\underset{Fe^{+2}}{\diagdown}}\!\!-\overset{Cys}{\diagup}$ (Cys_2-Fe^{2+}). This dipeptide was formed in aqueous solution by a substrate-dependent mechanism, involving DCDA-catalyzed peptide bond formation (Steinman *et al.*, 1965; Or'o and Stephan-Sherwood, 1976) between two cysteines in the presence of Fe^{2+} and ONPG. Its structure was carefully studied and elucidated by mass spectrometry, amino acid analysis, inductively coupled plasma (ICP) atomic adsorption and sequence analysis. The essential role of Fe^{2+} was demonstrated by the loss of both structure and activity upon treatment with H_2S. The Cys_2-Fe^{2+} – dipeptide, purified by HPLC, was shown to specifically hydrolyze ONPG into ONP and galactose, in a catalytic manner, with a specific activity only ~1000 times lower than the present day huge β-galactosidase enzyme.

We thus postulated that formation of catalytic peptides around their substrates as templates may have been a necessary step in catalyst formation prior to the appearance of the complex DNA/RNA based genetic and ribosomal machinery of "modern" protein synthesis.

In the present paper we propose, based on molecular mechanics computations, a model which describes the structural details of Cys_2-Fe^{2+} and its interactions with ONPG. Based on this model we offer an explanation why the presence of ONPG is essential for the formation of the catalyst. Furthermore, we demonstrate experimentally that this dipeptide can not only catalyze ONPG hydrolysis but also its formation *from its constituents*, ONP and galactose, using an immobilized form of the catalyst in a water-free environment. Obviously, the same enzyme, when hydrated, hydrolyzed ONPG to ONP and galactose.

2. Materials and Methods

2.1. Materials

Eupergit C, (EC) epoxy-activated polymethylmethacrylamide 150 μm beads, was a generous gift from Degussa Rohm Pharma Polymers (Darmstadt). Controlled-pore aminopropyl glass beads (CPG, 100 μm) were a generous gift from CPG (Corning, NY). ONPG and ONP were obtained from Sigma (St. Louis, MO). All the reagents used for synthesis were of analytical grade. The 100% ethanol used in the synthesis of ONPG was dried on a 5-Å molecular sieve (Aldrich Chemical Company, Milwaukee, WI) prior to use. β-D[1-^{14}C]-galactose (55 mCi/mmole, 50 μCi) was obtained from Amersham Biosciences (Uppsala). HPLC analyses were performed with HPLC-grade reagents (Merck, Darmstadt).

2.2. Preparation of the soluble catalyst Cys_2-Fe^{2+}

Cys_2-Fe^{2+} was prepared according to the procedure described previously (Kochavi *et al.*, 1997) with minor modifications: L-cysteine (33 mM) in 2 mL of 0.1 M

phosphate buffer, pH 7.0, containing DCDA (100 mM), ONPG (3.3 mM), KCl (0.1 M), $MgSO_4$ (1 mM) $FeSO_4$ (10 mM) and 2-mercaptoethanol (2-ME, 23 mM), was incubated in a 3 mL paraffin-sealed plastic cuvette at 50 °C for 72 h. The formation of Cys_2-Fe^{2+} was determined by following the increase in ONPG-cleaving activity, quantified by the increased absorbance at 420 nm of the ONP generated. The resulting Cys_2-Fe^{2+} was purified by RP-18 chromatography as described previously (Kochavi *et al.*, 1997). In a control experiment, cysteine was omitted from the reaction mixture and the rate of spontaneous hydrolysis of ONPG was measured (Table I).

2.3. Preparation of the immobilized catalyst

Stage (a)

EC: Since the epoxy groups of EC react with both amino and SH moieties, we coupled to the beads cystine which was reduced to cysteine by treatment with 2-ME after being coupled to the beads. EC beads (5 g dry weight) were extensively washed first with 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (phosphate buffered saline, PBS) and then with 0.1 M sodium carbonate buffer, pH 11. A solution of L-cystine (100 mg in 10 mL of the same buffer) was added to the beads and the mixture was incubated at room temperature for 24 h, during which 100% of the cystine was coupled to the beads as determined by measuring the absorbance at 250 nm of the supernatant. The beads were then extensively washed with PBS, washed once with 10 mL of 0.2 M 2-ME and incubated with 50 mL of the same

TABLE I

ONPG hydrolyzing activity of immobilized Cys_2-Fe^{2+} and its derivatives

Derivative	Activity (μmol/mL/48 h)
Spontaneous hydrolysis	0.15
Eupergit C-bound catalysts	
Eupergit-C blank	0.15
Cys-Cys	0.62
Cys-Cys-EE	0.55
Cys-Cys-SE	0.15
Cys + Z-Cys	0.18
Cys (Null)	0.19
Controlled pore glass beads	
CPG blank	0.16
Cys-Cys	0.37
Cys-Cys-EE	0.35
Cys (Null)	0.22

solution for 16 h. This operation resulted in cleavage of the S–S bonds and the generation of cysteine residues on the matrix, as well as blockage of the unreacted epoxy groups of the EC.

CPG: In contrast to the binding of cystine to the EC beads, L-cysteine was coupled directly to the amino glass beads by treatment with glutardialdehyde since this reagent reacts exclusively with amino groups. CPG beads (0.5 g dry weight) were incubated at room temperature with a 2 mL of freshly-prepared 2.5% glutardialdehyde in 0.2 M sodium bicarbonate buffer, pH 9.0, for 1 h. The beads were then washed with the same buffer and 12 mg of cysteine, in 3 mL of the buffer, were added. During 24 h of incubation at room temperature, 100% of the cysteine was coupled to the beads as determined by reaction of aliquots of the supernatant with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Riddles *et al.*, 1983). The beads were then washed with PBS and incubated with 3 mL of 1 M 2-ethanolamine in water to block all nonreacted aldehyde groups.

At the end of the first stage we had a SH-free cysteine fixed to each of the carriers via its amino moiety at concentrations of 160 (assuming one immobilized cysteine generated by the reduction of the cystine molecule) and 100 μmol/g beads for the EC and CPG preparations, respectively.

Stage (b)

Samples of the two immobilized cysteine preparations (0.8 g, wet weight, each, equivalent to approximally 200 mg dry weight) were suspended in 3 mL of 0.1 M phosphate buffer, pH 7.0 containing L-cysteine (33 mM), ONPG (3.3 mM), DCDA (100 mM), KCl (100 mM), $MgSO_4$ (1 mM) $FeSO_4$ (10 mM) and 2-ME (23 mM). The suspension was placed in a paraffin-sealed plastic cuvette and incubated at 50 °C for 96 h. The activity of the formed Cys_2-Fe^{2+} was determined by following the increase in absorbance at 420 nm of ONP generated upon ONPG hydrolysis. In control experiments, cysteine was replaced by L-cysteine ethyl ester (Cys-EE), S-ethyl L-cysteine (Cys-SE), carbobenzoxy-L-cysteine (Z-Cys) or none. These complexes were extensively washed with PBS and stored at 4 °C until further used.

This lengthy procedure was adopted since an attempt to prepare the catalyst by the classical Merrifield method, in the presence of Fe^{2+} ion, and in the absence of ONPG failed to produce the catalyst Cys_2-Fe^{2+}, leading instead to the formation of S–S oxidized Cys-Cys. This illustrates the *mandatory* requirement of the presence of ONPG to promote the formation of the catalyst.

2.4. Activity measurements

To determine ONPG hydrolysis by the immobilized catalysts, 100 mg (wet weight) of each catalyst were suspended in a 3 mL paraffin-sealed plastic cuvette with 2 mL of 0.1 M phosphate buffer pH 7.0, containing ONPG (3.3 mM), KCl (0.1 M), and $MgSO_4$ (1.0 mM) and agitated at 50 °C for 24 h. ONPG hydrolysis was followed by measuring the increase with time in absorbance of ONP at 420 nm.

2.5. Application of the immobilized catalysts to the synthesis of ONPG

The immobilized catalysts, EC-Cys_2-Fe^{2+} and CPG-Cys_2-Fe^{2+}, were used for the synthesis of ONPG under anhydrous conditions. The EC derivative was used for non-radiolabeled synthesis while with the CPG derivative radiolabeled galactose was included in the reaction mixture to produce ^{14}C-labeled ONPG.

EC-Cys_2-Fe^{2+} beads

Hundred milligrams of these beads were extensively washed with 100% dry ethanol prior to use. ONP (3.3 mM) dissolved in 2 mL of dry ethanol and 20 mg of β-D-galactose as powder (corresponding to 55 mM upon full dissolution) were added. The reaction mixture was placed in a paraffin-sealed 3 mL plastic cuvette and agitated for 24–72 h at 50 °C. Then the beads were removed by centrifugation and the supernatant was evaporated in a Speed–Vac apparatus (Savant-GMI, Albertville, MI). After dissolution in 0.5 mL of 0.1% TFA in water, the sample was applied to a RP-18 HPLC column (Vydak, Hesperia, CA) which was developed in a Gilson (Viler Le Bell, France) Model 302 HPLC at 1 mL/min with increasing acetonitrile concentrations, using the same gradient profile as the one depicted in Figure 4. Absorbance at 220 nm was followed using a LKB Diode Array Detector (Amersham-Pharmacia, Uppsala).

CPG-Cys_2-Fe^{2+} beads

Hundred milligrams of beads were extensively washed with dry ethanol prior to use. The reaction mixture was prepared as follows: Radiolabeled ^{14}C-galactose (5×10^6 cpm, corresponding to 0.1 μmol), was first mixed with 20 mg D-galactose in 100 μL of water in a 3 mL plastic cuvette (yielding a specific activity of 75 CPM/mole of lactose) and dried by evaporation in a Speed-Vac apparatus. ONP (3.3 mM), dissolved in 2 mL of dried ethanol and 100 mg of CPG-Cys_2-Fe^{2+} beads were added to the cuvette that was sealed with paraffin and agitated for 72 h at 50 °C. Then, the supernatant was recovered, diluted 200 fold with water to obtain a final concentration of 0.5% ethanol and applied to a RP-18 Sep-Pak (Waters, Milford, MA) cartridge. The cartridge was extensively washed with water and then with 100% ethanol, collecting 2 mL fractions. ^{14}C-labeled compounds in each fraction were detected using a Beckman–Coulter (Fullerton, CA) beta counter. The ethanol fractions were pooled, evaporated, re-dissolved in 0.5 mL of 0.1% TFA in water and applied to a RP-18 HPLC column for the analysis of ONPG formed in the reaction.

2.6. Molecular modeling of S–S oxidized and Fe^{2+} coordinated Cys_2

Models of Cys_2-Fe^{2+} and Cys_2-(S–S) were constructed *by computer simulation* with the Insight package (Acers Inc., San Diego, CA). The model structure of the

S–S oxidized Cys-Cys dipeptide was energy minimized using the Discover module of Insight, with the Amber force field. Then 30,000 dynamics steps were performed (1 femtosecond long each) at 350 °K and finally the minimization was repeated until the RMS (root mean square) force field derivative was less than 0.001. The structure of Cys_2-Fe^{2+} was determined as follows: we assumed that the two SH groups bind to the Fe^{2+} ion and tested different coordination modes: an octahedral coordination (with either 90 or 180 degrees between the putative Fe–S bonds) and a tetrahedral coordination. Then the structure of the dipeptide was subjected to dynamics and energy minimization, as described above. In these computations the Fe^{2+} ion was only a point in space, which helped to constrain the positions of the sulfur atoms in the Cys-Cys peptide. Notably, two starting conformations of the peptide bond in the Cys-Cys peptide, *trans* and *cis*, were used in each set of computations, with and without Fe^{2+}. Next, we tested several possibilities of binding ONPG to the Cys_2-Fe complex, assuming that a stable structure will be formed when at least 3 of the ONPG oxygens interact with Fe^{2+}, and requiring that the minimized structure of ONPG under these constraints is in accord with commonly accepted bond geometry.

3. Results

3.1. The structure of the Cys_2-Fe^{2+}-ONPG complex

Previously (Kochavi *et al.*, 1997) we have demonstrated that a catalytically-active dipeptide Cys_2-Fe^{2+} can be formed by DCDA-assisted condensation of two cysteins and a Fe^{2+} ion only in the presence of ONPG, which, in turn, serves as a substrate for this catalyst. In contrast, in the absence of ONPG, Cys_2-(S–S), is formed. This dipeptide, devoid of Fe^{2+}, does not possess any catalytic activity towards ONPG.

The first goal of the present work was to reveal the mechanism of the substrate-directed formation of Cys_2-Fe^{2+}: what are the molecular details of the Cys_2-Fe^{2+} – ONPG interaction and how does the presence of ONPG direct the formation of an *S-Fe*$^{2+}$, rather than *S–S* bond. A second goal was to demonstrate the ability of the catalyst to catalyze the formation of ONPG from ONP and galactose under anhydrous conditions.

First we analyzed the energy minimized model structures for the two compounds – Cys_2-(S–S) and Cys_2-Fe^{2+}. We found that the two dipeptides differ in the conformation of their peptide bonds. While the peptide bond in Cys_2-(S–S) always reached the more common *trans* conformation, the peptide bond in Cys_2-Fe^{2+} reached a *cis* conformation, regardless whether the starting conformation was *cis* or *trans* (Figures 1A and 1B). Our finding that Cys_2-Fe^{2+} is formed only in presence of ONPG suggests that complexing of the cysteines with the substrate via the Fe^{2+} ion affects the conformation of the resulting peptide bond of the dipeptide formed by DCDA. Molecular modeling was further used to obtain a putative

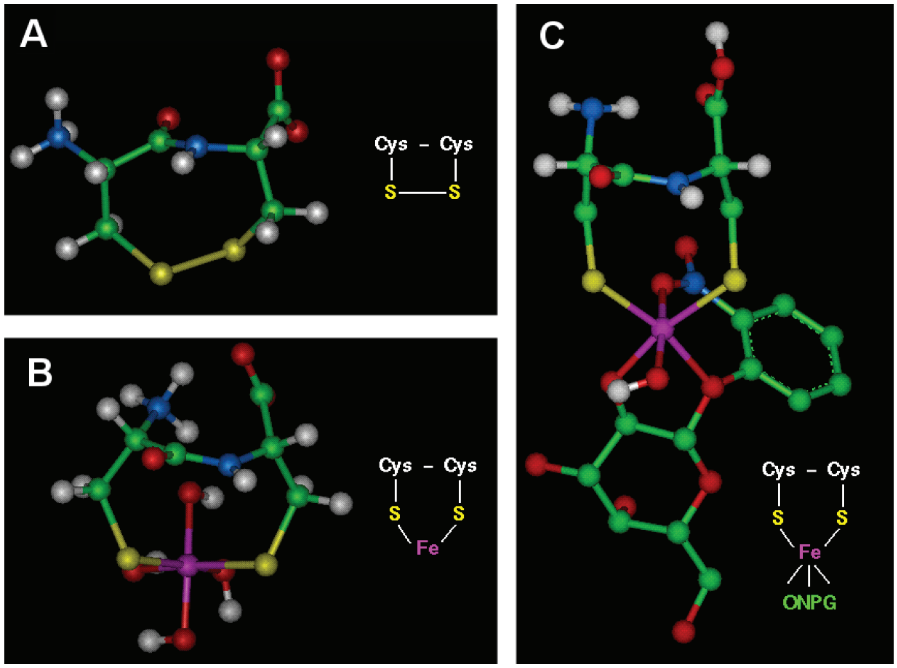

Figure 1. Model structures of Cys_2-(S—S) (A) and Cys_2-Fe^{2+} (B) and of the complex between Cys_2-Fe^{2+} and ONPG (C). The models were constructed using the Insight Program by thermal simulation as described in Section 2. The atoms are represented by the following color-code: Green-Carbon; Blue-Nitrogen; Red-Oxygen; Grey-Hydrogen; Yellow-Sulphur and Purple-Iron.

structure for the complex between Cys_2-Fe^{2+} and ONPG (Figure 1C). The model suggests that the Fe^{2+} ion of Cys_2-Fe^{2+} is coordinated to three oxygen atoms of ONPG, two of galactosyl hydroxyls and one of the nitro groups of the nitrophenol. A water molecule may complete the octahedral coordination of Fe^{2+}. The predicted structure requires proximity between the nitro group and the hydroxyl at position 2 of the galactose ring. Our previous finding that Cys_2-Fe^{2+} specifically cleaves the *ortho* conformer ONPG but not the homologous *para* conformer (PNPG, Kochavi *et al.*, 1997), strongly supports this hypothesis, as the NO_2 moiety at the *para* conformation is oriented further away from the galatosyl hydroxyls and cannot react with the attached Fe^{2+} ion. The electron withdrawal from ONPG oxygens by the interaction with the Fe^{2+} ion presumably renders the glycosidic bond between ONP and galactose less stable and hence the hydrolytic activity towards ONPG observed for the catalyst.

This model suggests a preferable interaction of the Fe^{2+} ion with the galactose OH at position 2. This is supported by experimental evidence showing that Cys_2-Fe^{2+} was not active towards the ONPG analogue o-nitrophenyl 2-N-acetyl-D-galactoside (data not shown).

3.2. THE IMMOBILIZED Cys_2-Fe^{2+} PREPARATIONS AND THEIR CATALYTIC ACTIVITIES IN ONPG HYDROLYSIS

As mentioned above, our second goal in the present study was to demonstrate the ability of Cys_2-Fe^{2+}, which has been generated in the presence of ONPG, to catalyze not only its cleavage but also its formation from D-galactose and o-nitrophenol. Obviously, this anabolic reaction, which involves the elimination of a water molecule, can proceed *only* under anhydrous conditions, e.g. in the presence of 100% ethanol. However, under such conditions, it is impossible to keep the dipeptide in solution and in an active form. In order to preserve the structure and the activity of the peptide in the absence of water, water-insoluble derivatives of the catalyst were prepared by attaching it to insoluble matrices. Such treatment has been shown in the literature to sustain the activity of many enzymes under anhydrous conditions (for a recent review see Klibanov, 2001). By replacing cystein with its analogs in stage (b) of the synthesis (see Section 2 for details) several derivatives of the immobilized dipeptide were obtained. These were tested for their ability to hydrolyze ONPG. As shown in Table I, addition of Cys or Cys-EE as the second soluble moiety, yielded active immobilized compounds, while addition of Cys-SE or Z-Cys failed to produce active compounds. Presumably, the N-terminally-blocked Z-Cys was incapable of forming a peptide bond with the immobilized cysteine, while Cys-SE could form a peptide bond but could not bind the Fe^{2+} ion. These findings demonstrate that peptide bond formation between the two cysteine residues and the existence of free SH groups on both cysteines are essential for formation of an active catalyst. The low activity observed for a single cysteine attached to the matrices parallels the activity of free cysteine in solution, as previously reported (Bar-Nun *et al.*, 1994).

Calculation of the amount of active material immobilized on the beads was a difficult task: although the amount of the first cysteine on each matrix was easily determined by reaction with DTNB or absorbance measurements, as described in Section 2, the amount of the second cysteine, as well as that of Fe^{2+} determined by ICP could not be indicative of the amount of the active dipeptide because (a) most of the second cysteine form oxidized di cysteine (S—S) which cannot be detected by DTNB and is anyhow not active, and (b) both matrices adsorbed non-specifically considerable amounts of iron ions. Since the dipeptide was attached to the beads by a covalent, non-cleavable bond, it was not possible to remove it for analysis, as was carried out previously with the soluble catalyst. One should also bear in mind that only small amounts of active Cys_2-Fe^{2+} are expected to be formed on the beads, with higher amounts of beads-cystein and beads-Cys_2 expected.

The amount of immobilized Cys_2-Fe^{2+} could be, however, estimated from its activity. Assuming that the specific activity of the immobilized Cys_2-Fe^{2+} is similar to the previously determined specific activity of the soluble catalyst (0.06 mM ONP/mg catalyst/h, Kochavi *et al.*, 1997) the amounts of matrix-attached catalysts

were calculated as 0.36 and 0.17 mg catalyst/g matrix or 1.7 and 0.7 μmol catalyst/g matrix for the EC- and CPG- derivatives, respectively.

3.3. Synthesis of ONPG from ONP and galactose using the immobilized Cys_2-Fe^{2+} preparations

The ability of the immobilized Cys_2-Fe^{2+} to catalyze the synthesis of ONPG was first examined with the EC preparation, which was incubated with ONP and D-galactose in dry ethanol. D-Galactose, which has a very limited solubility in ethanol, was added as a powder and was expected to be taken in as the reaction proceeded. Within three days of incubation at 50 °C, a time dependent formation of ONPG was observed when aliquots of the reaction mixture were analyzed by RP-18 chromatography (Figure 2). In a control experiment, using a blank EC preparation under the same conditions, no formation of ONPG was detected. In addition to ONPG another highly hydrophobic, yet unidentified, material was produced in the reaction mixture containing the immobilized catalyst.

In order to ensure that the ONPG that appeared in the reaction mixture of the EC-coupled catalyst was *de novo* synthesized and was not resulted by a slow release of residual ONPG introduced earlier during the catalyst preparation stage, we examined the *de novo* synthesis of radiolabeled ONPG by the catalyst from its constituents ^{14}C-D-galactose and ONP. This was carried out with the CPG-immobilized catalyst under the same conditions as used with the EC-catalyst. As shown in Figure 3, under these conditions, a radiolabeled hydrophobic Sep-Pak RP-18-bound fraction was produced by the immobilized catalyst but not when the immobilized catalyst was replaced by blank CPG beads. In a RP-HPLC analysis this hydrophobic fraction was found to contain about 20% radiolabeled ONPG (Figure 4). The remaining radiolabeled material appeared to be a more hydrophobic material, the nature of which is still obscure. Its production only in the presence of Cys_2-Fe^{2+} and the lack of UV absorbance at 220 nm suggests that this fraction may represent oligosaccharide-iron complexes formed under anhydrous condition, which may adhere to the silica groups of the column. Formation of such adducts in methanol in the presence of Na^+ ions has been studied by Rao *et al.* (Geetha *et al.*, 1995; Rao *et al.*, 2000).

4. Discussion

Life depends on biochemical processes which are carried out and regulated by enzymes, catalyzing a variety of anabolic and catabolic reactions. These are produced in the biological world by the complex ribosomal machinery according to the genetic code instructions. Obviously, in the absence of this complex machinery, prebiotic "enzymes" should have been evolved by much simpler chemical reactions, presumably involving template-assisted mechanisms (Bar-Nun *et al.*,

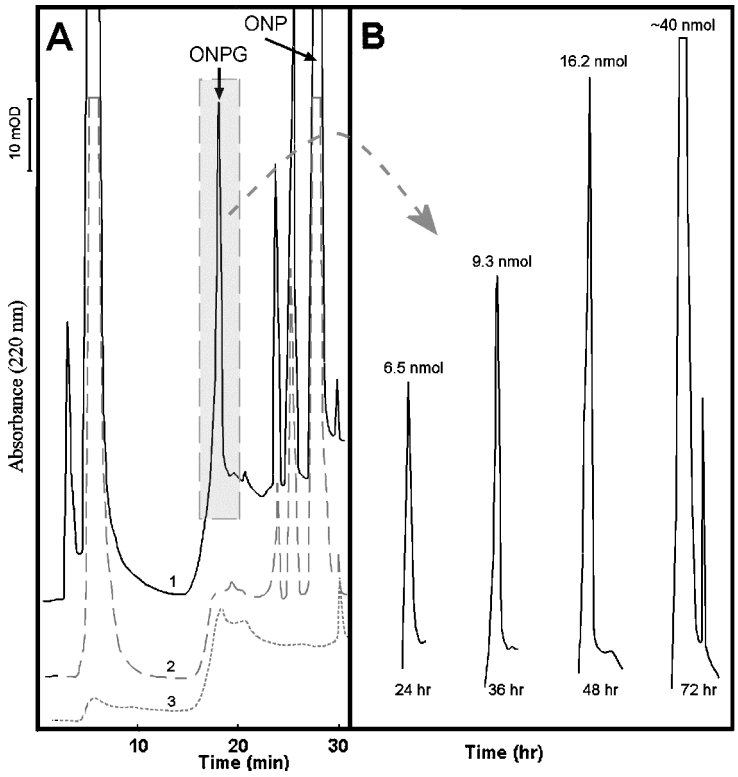

Figure 2. HPLC chromatography of the reaction products formed by incubation of ONP, β-D-galactose and Eupergit-C-immobilized Cys_2-Fe^{2+}. In frame A the complete chromatogram obtained after 36 h of incubation is shown. The chromatogram in curve 1 was obtained for the reaction with the EC-bound Cys_2-Fe^{2+}, where ONPG is formed. Curve 2 was obtained with blank Eupergit C and curve 3 is a control buffer. The elution times of ONPG and ONP were determined with authentic markers as shown at the top of the figure. In frame B the peaks corresponding to ONPG are depicted after 24, 36, 48 and 72 h. The values for ONPG formation were calculated after integration using a molar extinction coefficient of 6670 M^{-1} for ONPG in 0.1% TFA and 8% acetonitrile in water at 220 nm (determined by us with authentic ONPG) and multiplied by a dilution factor of 2.5. Calculation of the peak area at 72 h, which overshoot the scale, was made by extrapolation.

1994; Lahav and Nir, 1997; Harris *et al.*, 1999). It is widely-accepted that catalytic RNA molecules, the ribozymes, preceded "modern" enzymes in catalyzing phosphoryl transfer reactions and even peptide bond formation, often using metals and/or amino acids as cofactors (Szathmary, 1999; De-Rose, 2002). However, ribozymes were too big to have served as the catalysts during the earlier stages of chemical evolution. The prebiotic catalysts, peptides or oligonucleotides, were supposedly simple molecules, bearing active functional groups catalyzing specific reactions. Previously, we have suggested that substrate-dependent synthesis of catalytic peptides may have served in generating specific "mini enzymes" during the prebiotic era. This was demonstrated experimentally by the formation of an active

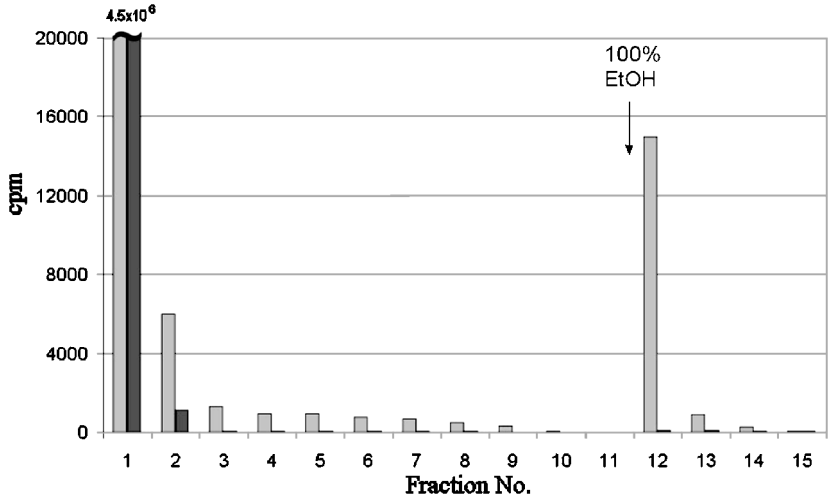

Figure 3. Sep-Pak RP-18 solid phase extraction of the reaction products obtained after incubation of ^{14}C-galactose and ONP with the CPG-bound Cys_2-Fe^{2+} under anhydrous conditions. Bars of curve 1 (light grey) were obtained with CPG-Cys_2-Fe^{2+} and those of curve 2 (dark grey) with blank CPG. Fractions 1–11 were eluted with water and fractions 12–15 (after the arrow) with 100% (absolute) ethanol.

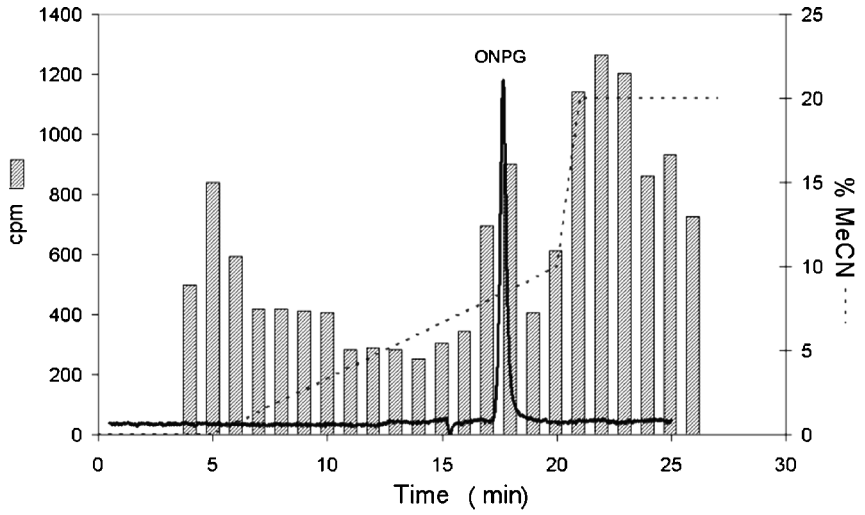

Figure 4. HPLC separation of the hydrophobic fraction of reaction products obtained after incubation of ^{14}C-galactose and ONP with the CPG-bound Cys_2-Fe^{2+} (fraction 12 in Figure 3) on a RP-18 column. The radioactivity in each fraction was measured. The elution profile of authentic ONPG is shown. The broken line represents the gradient curve of acetonitrile. The more hydrophobic fractions, eluting after ONPG, are presumably galactose-iron complexes as explained in the text.

metallo-dipeptide, Cys_2Fe^{2+} which efficiently hydrolyzes ONPG but not PNPG (Kochavi *et al.*, 1997). The mandatory requirement for the presence of the substrate and Fe^{2+} ions, led us to suggest that peptide bond formation in the catalyst is directed and facilitated by the substrate, coupled to the cysteines via the Fe^{2+} ion. This mechanism may be analogous to other template-assisted peptide bond formation reported in the literature such as the enhancement of carbonyldiimidazole assisted Cys-Cys peptide bond formation by Cys-containing peptides scaffolds (Chu and Orgel, 1999) or the Cu^{2+}/NaCl catalysis of amino acids dimerization by salt induced peptide formation (SIPF, Schwendinger and Rode, 1992). The molecular modeling analysis performed in the present work suggested a detailed structure for the Cys_2Fe^{2+} and its complex with ONPG via three coordinations of the Fe^{2+} ion. This analysis showed that Fe^{2+} complexation by the two cysteine induces a rare *cis* conformation for the peptide bond in the catalyst, opposite to the common *trans* conformation observed for peptide bond in (S─S)-Cys_2 as was previously obtained by the Merrifield synthesis.

The high energy barrier between the *cis* and *trans* conformations, resulting from the partial double bond nature of the peptide bond and estimated to be from 16–18 kcal/mol (Perricaudet and Pullman, 1973) up to 22 kcal/mol (Fersht, 1971) hinders the spontaneous *cis/trans* transformation thus maintaining the catalyst structure and activity. Because of this high energy barrier, *cis/trans* transformation in the living cell are catalyzed by specific isomerases. Moreover, the *cis/trans* transformation of certain peptide bonds in "modern" enzymes often possesses a crucial role in regulating their activities (for a recent review see Fischer and Aumuller, 2003).

The above model suggests a complex formation between two cysteines, Fe^{2+} ions and ONPG. Association of metals, including iron ions, with saccharides to form mono-, oligo- and polynuclear complexes have been widely reported in the literature (for a recent review see Gyurcsik and Nagy, 2000). Such metal-saccharide interactions often occur via the weak chelating power of the sugar hydroxyls. A

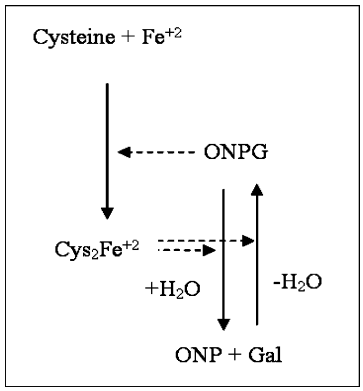

Figure 5. The formation of Cys_2Fe^{2+} on ONPG and its catalytic activities in the hydrolysis and synthesis of ONPG under hydrous and anhydrous conditions, respectively.

$\log K = 3.09$ was reported for the interaction of Fe^{2+} with galacturonic acid (Escandar *et al.*, 1990) while $\log K = 3.89$ was reported for the reaction of the sugar with Fe^{3+} (Deiana *et al.*, 1989). We assume that the cysteine-complexed Fe^{2+} should interact with ONPG in a similar manner.

In the present work we have shown that Cys_2Fe^{2+} was not only able to hydrolyze the substrate, but also to generate it from its components, ONP and galactose. The ability of the catalyst to synthesize the substrate that mediates its own formation creates an autocatalytic cycle where ONPG catalyzes the formation of a catalyst which, in turn, catalyzes ONPG formation. This autocatalytic cycle can only operate by switching between high and low water activity conditions (Figure 5), such as in tidal pools cycling between wet and dry environments. Indeed, lowering water activity by increased salt concentration in evaporating tidal pools has been suggested to be an important prebiotic catalytic factor in another water elimination reaction, the formation of peptides (Saetia *et al.*, 1993, Schwendinger and Rode, 1992).

5. Conclusions

1. In this work we studied further the formation of a catalytic peptide, Cys_2Fe^{2+}, around its substrate ONPG. This "mini enzyme" was shown to catalyze the hydrolysis of ONPG to ONP and galactose. It was also shown to catalyze the formation of ONPG from its constituents under anhydrous conditions. This was achieved by synthesis of the "mini enzyme" in an immobilized form on insoluble matrices – Eupergit C and glass beads. We show that an *autocatalytic cycle* can be obtained by these reactions by cycling between wet and dry conditions such as in tidal pools.
2. By molecular modeling we have shown that in the Cys_2Fe^{2+}-ONPG complex the six coordinations of the Fe^{2+} are connected to the two S atoms of the peptide, two oxygens of the galactose hydroxyls, one of the NO_2 oxygens and one hydroxyl of a water molecule. The Fe^{2+} complexation by the two cysteine induce the rare *cis* conformation for the peptide bond rather than the *trans* conformation observed for (S–S)-Cys_2 as was previously obtained by the Merrifield synthesis.

Acknowledgments

We thank the anonymous reviewers for their most helpful suggestions.

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