Two coupled enzymes perform in parallel the 'AND' and 'InhibAND' logic gate operations

Ronan Baron, Oleg Lioubashevski, Eugenii Katz, Tamara Niazov and Itamar Willner*

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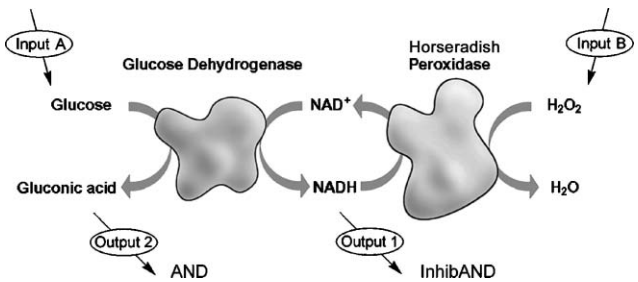
The coupled activation of two enzymes: glucose dehydrogenase (GDH) and horseradish peroxidase (HRP), is used to construct the parallel-operating AND and InhibAND logic gates. The added substrates for the respective enzymes, glucose and H_2O_2, act as the gate inputs, while the biocatalytically generated NADH and gluconic acid provide the output signals that follow the operations of the gates. The two gates are generated in the same vial, thus allowing the logic operations to take place in parallel, and the simultaneous readout of the functions of the gates.

Substantial research efforts are directed towards the development of molecule- and biomolecule-based logic gates.[1,2] For example, molecular assemblies that use optical readout of chemical and light inputs enabled the construction of different AND gates.[3,4] Also, the photoisomerization of a quinone-functionalized monolayer with the parallel input of a pH signal was employed to construct an AND gate that allowed the electrochemical readout of the gate function.[5] Similarly, molecule-based XOR or InhibAND logic gates were designed.[6] The parallel operation of different gates was used to develop systems capable of performing arithmetic operations. For example, a molecular structure that exhibited XOR and AND logic gate functions was used to construct a half-adder system,[7] and a molecular assembly that performed InhibAND and XOR operations was employed to construct a half-subtractor.[8] In analogy, biomolecules were used as computing elements.[9] For example, gene-based artificial circuits that perform a bistable toggle switch function were developed.[10] Also, DNA–enzyme systems were coupled to perform programmable biochemical transformations that mimicked the basic computing of finite automatons that logically analyzed the content of RNA, and generated a product that acted as an output that controlled the levels of gene expression.[11] The use of enzymes as computing elements is, however, scarce. For example, a XOR gate was constructed using the dynamic conformational perturbation of malate dehydrogenase in response to added ions,[12] and an AND gate was generated by the application of a structurally modified enzyme and its inhibitor.[13] The use of enzymes as logic gate components is particularly attractive as numerous biocatalysts require two co-substrates for their activation, *e.g.*, a substrate–cofactor pair or substrate–O_2 for an oxidase. Furthermore, the possible regulation of enzymes by other stimuli such as inhibitors, pH or temperature, paves the way to regulating the biocatalysts by different input signals, thus allowing the design of different gates. In the present study we demonstrate the use of two coupled enzymes for the construction of an AND and an InhibAND gate.

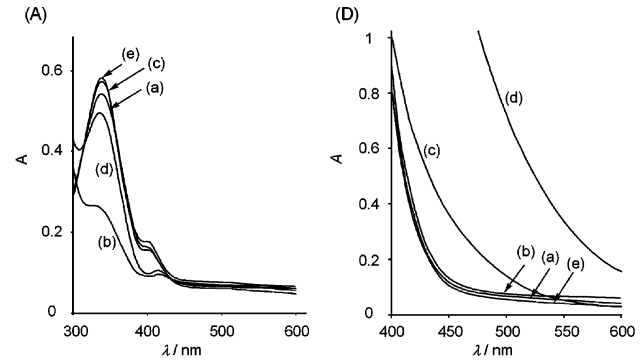
For the construction of the logic gates, we have employed two redox enzymes and their substrates as the input units. The computational functions were read out by optical means (Scheme 1). The two enzymes are glucose dehydrogenase and horseradish peroxidase, and the two chemical inputs are glucose (input A) and hydrogen peroxide (input B). Moreover, the cofactor NADH was added to the system, and it acts as a 'bridging relay' between the two biocatalysts. The outputs of the biocatalytic logic gates are read out in terms of the absorbance change at the appropriate wavelength. For each enzymatic logic gate, two distinct regions for the absorbance changes, $|\Delta A|$, are defined. One region of the absorbance change that is going from 0 to 0.15 a.u. was defined as the low level output and corresponds to a logical '0'. A second region of the absorbance change which is going from 0.25 to 1 a.u. was defined as a high level output, and corresponds to a logical '1' state.†



Scheme 1 Schematic parallel operation of the coupled two-enzyme system that performs the AND and InhibAND logic gate functions.

The InhibAND gate is generated by reading out the concentration of NADH in the system after the biocatalytic enzymatic reaction was allowed to take place for 20 minutes. To obtain this gate, NAD^+ is initially absent in the system. The output corresponds to the variation of the absorbance of NADH followed spectroscopically at $\lambda = 340$ nm, (Scheme 1, output 1). When both inputs are FALSE, configuration (0,0), no significant absorbance variation is measured at 340 nm (Fig. 1(A), curve (a)) leading to a FALSE output. Indeed, as previously reported in the literature,[14] NADH was found to be stable enough at pH 6.8 within the timescale of our experiment. If input A is FALSE and input B is TRUE, configuration (0,1), a clear decrease in the absorbance at 340 nm, that results in a TRUE output, is observed (curve (b)). This observation shows that, in these conditions, the biocatalytic oxidation of NADH leads to a decrease in the concentration of NADH. When input A is TRUE and input B is FALSE, configuration (1,0), no change is observed in the absorbance at

Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: willnea@vms.huji.ac.il; Fax: 972-2-6527715; Tel: 972-2-6585272



(C)

Input A	Input B	Output InhibAND
0	0	0
0	1	1
1	0	0
1	1	0

(F)

Input A	Input B	Output AND
0	0	0
0	1	0
1	0	0
1	1	1

Fig. 1 (A) Absorbance features of the InhibAND gate. (B) Bar presentation of the InhibAND gate absorbance outputs. (C) Truth table corresponding to the InhibAND gate. (D) Absorbance features of the AND gate. (E) Bar presentation of the AND gate absorbance outputs. (F) Truth table for the AND gate. For both spectra, inputs correspond to: (a) (0,0), (b) (0,1), (c) (1,0), (d) (1,1) and curve (e) corresponds to the absorbance of the biocatalytic system prior to activation by the inputs. For both bar presentations, the regions of output '0' and output '1' are indicated.

340 nm and the system provides a FALSE output (curve (c)). In this case the system lacks NAD^+ and the oxidation of glucose cannot proceed. When both inputs are TRUE, configuration (1,1), a low change of absorbance is observed, giving a FALSE output (curve (d)). In this case, the enzymatic transformation of H_2O_2 occurs, resulting in a decrease of the concentration of NADH; however, the enzyme concentrations are balanced in such a way that the NAD^+ produced is rapidly reduced back to NADH during the biocatalytic oxidation of glucose by glucose dehydrogenase. The bar presentation of the modulus of the absorbance change is shown in Fig. 1(B). In summary, the two-enzyme system can perform the InhibAND logic operation that is TRUE only if input B is TRUE, in accordance with the truth table given in Fig. 1(C).

The AND gate is generated by reading out the concentration of gluconic acid in the system, under the same conditions that were employed for the InhibAND gate operation, namely, after the biocatalytic reaction was allowed to proceed for 20 minutes. The gluconic acid produced is reacted with hydroxylamine, and the hydroxamate produced is complexed with Fe(III) to yield a red colour.[15] The output corresponds to the variation of the absorbance of the hydroxamate–Fe(III) complex that was followed spectroscopically at $\lambda = 500$ nm, (Scheme 1, output 2). If no substrate is added to the system, both inputs are FALSE, and no absorbance change is measured at 500 nm, and thus, the output is FALSE (Fig. 1(D), curve (a)). When input A is FALSE and input B is TRUE, configuration (0,1), no gluconic acid is detected leading to a FALSE output (curve (b)). Here, the peroxidase reduces H_2O_2 and oxidizes NADH to NAD^+, but the lack of glucose prevents the formation of gluconic acid and the development of the colour. If input A is TRUE and input B is FALSE, configuration (1,0), a very low amount of gluconic acid is detected (curve (c)). This minute amount originated from the oxidation of glucose by glucose dehydrogenase which takes place due to the existence of small amounts of NAD^+ in the reaction system (this low amount of NAD^+ in the system is due to the spontaneous decomposition of NADH). When both inputs are TRUE, configuration (1,1), a large absorption change is observed at 500 nm, revealing that gluconic acid was generated (curve (d)). In the later configuration of the system, the NAD^+ necessary for the oxidation of glucose is provided by the oxidation of NADH by the peroxidase. The bar presentation of the modulus of the absorbance changes is presented in Fig. 1(E). That is, the AND logic gate operation, that yields a TRUE output only when both inputs are TRUE, was achieved (truth table given in Fig. 1(F)). We thus conclude that the two-enzyme system, described herein, allows the parallel operation of both InhibAND and AND logic operations.

Our study has demonstrated that a simple system consisting of two coupled native enzymes, glucose dehydrogenase and horseradish peroxidase, perform the AND and InhibAND logic gate operations *in vitro.* Furthermore, we emphasize that this system allows the reading of the two gates in 'one pot'. The availability of numerous enzymes that may be coupled (or operated in series) suggests that logic gate circuits of enhanced complexity may be envisaged. Moreover, an alternative to the optical readout of the operations of enzyme-based logic gates might be an electrical readout using electrically 'wired' enzymes with electrodes.[16] An electrical readout would considerably shorten the response time of the systems, and will allow the resetting of the systems. The use of enzymes and their substrates as the active components of logic gates has significant advantages over molecule-based logic gates. The availability of diverse enzymes, and the high catalytic functions of biocatalysts may exclude the need to synthesize complex molecular structures.

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Notes and references

† *Materials and methods*: all chemicals and enzymes were purchased from Aldrich or Sigma. The enzymes employed in the study include glucose dehydrogenase from *Thermoplasma acidophilum* (E.C. 1.1.1.47) and peroxidase from horseradish (E.C. 1.11.1.7). All measurements were performed at 25 ± 2 °C. A Shimadzu UV-2401PC UV–visible spectrophotometer was used to record the absorbance features of the samples. *Composition of the system*: the absorbance variations correspond to the differences in absorbance values before and after the enzymatic reaction was allowed to proceed. The inputs that were used for all of the coupled two-enzyme systems corresponded to added β-D-(+)-glucose, 1 M, (input A) and added H_2O_2, 0.1 M, (input B). The system is composed of a 250 μl solution of

4.5 U horseradish peroxidase, 12 U glucose dehydrogenase and 1×10^{-4} M NADH in 0.01 M phosphate buffer, pH = 6.8. For both gates the modulus of the absorbance change, $|\Delta A|$, was measured after a time interval of 20 min. This time interval was selected by following the kinetics of the operation of the logic gates, that revealed a tendency towards saturation of the absorbance values after this time interval. The deviation of the $|\Delta A|$ values did not exceed ±5%. Gluconic acid was detected colorimetrically using a procedure adapted from the literature.[15] First, 250 μl of a solution 1 (EDTA 5×10^{-3} M, TEA 0.15 M in water) and 25 μl of a solution 2 (NH_2OH 3 M in water) were added to the 250 μl solution that included the two-enzyme system, and the reaction was allowed to proceed for 15 min. Then, 125 μl of a solution 3 (HCl 1 M, $FeCl_3$ 0.1 M, CCl_3COOH 0.25 M in water) were added to the previous mixture and the reaction was allowed to proceed for 5 min. The above detailed procedure enables the quantitative detection of gluconate by following the resulting absorbance at 500 nm.

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