Enzymatic Computing

Klaus-Peter Zauner and Michael Conrad*

Department of Computer Science, Wayne State University, Detroit, Michigan 48202

The conformational dynamics of enzymes is a computational resource that fuses milieu signals in a nonlinear fashion. Response surface methodology can be used to elicit computational functionality from enzyme dynamics. We constructed a tabletop prototype to implement enzymatic signal processing in a device context and employed it in conjunction with malate dehydrogenase to perform the linearly inseparable exclusive-or operation. This shows that proteins can execute signal processing operations that are more complex than those performed by individual threshold elements. We view the experiments reported, though restricted to the two-variable case, as a stepping stone to computational networks that utilize the precise reproducibility of proteins, and the concomitant reproducibility of their nonlinear dynamics, to implement complex pattern transformations.

Introduction

Enzymes possess a powerful intrinsic capacity to recognize specific molecules in a complex milieu. Conformational dynamics play an important role. These dynamics are influenced both by the specific molecules recognized, most prominently the substrate, and by milieu features that are less specific (such as ionic composition). The protein conformational dynamics integrates or fuses all of these conformational effects to modulate catalytic activity. We can think of the enzyme as a molecular pattern recognizer relative to molecules it complexes with on the basis of specific shape and as a signal pattern recognizer relative to the milieu features.

The pattern recognition activity can be phrased in computational terms. Enzymes are context-sensitive pattern recognizers that respond to both specific molecular shapes and to the chemical environment. The mode of processing is very different from digital switching technology, which is essentially context-free. The underlying physics of conformational interaction allows for complex effects that could contribute to cellular information processing and may find use in potential molecular computing technologies.

Our interest here is in what kind of information processing operations an enzyme can perform in response to milieu context. The general approach is first to utilize a response surface to phenomenologically characterize the effect of variation in selected milieu features on enzyme activity. The milieu features can be interpreted as input signals and the response surface as the input-output function implemented by the enzyme in the range under consideration. The response surface can then be analyzed to determine whether a given signal processing operation is possible in this range. We have constructed a tabletop setup to study these signal processing operations in a device-like context.

We applied the above approach to pig heart mitochondrial malate dehydrogenase (MDH). MDH catalyzes the reaction of L-malate and NAD^+ to oxalacetate, NADH, and a proton. The reaction was monitored by spectroscopic detection of NADH. Mg^{2+} and Ca^{2+} were selected as the variable milieu features. The response surface shows that Mg^{2+} and Ca^{2+} can be used to implement a variety of two-bit pattern classifications. Of most interest from a computational point of view are those operations that cannot be accomplished by linear summation. The work to be reported here focused on the exclusive-or (XOR) operation, since this is an operation that cannot be performed by a linear element (e.g., by neurons typically used in artificial neural nets or by transistors). The XOR is the simplest example of a linearly inseparable pattern recognition problem, i.e., a problem in which the patterns cannot be classified with a single threshold or a single layer perceptron. This is the class of problems that Minsky and Papert showed could not be solved by a perceptron (*1*).

Simulation studies conducted by Arkin and Ross have shown that enzymatic implementation of the XOR operation is theoretically possible (*2*). Our experiments show that MDH can perform the XOR in response to Mg^{2+} or Ca^{2+} used either alone or in combination as signal carriers. The activity first increases to a maximum and then decreases as the concentration of ion decreases. Thus the response surface is convex (or strictly nonmonotonic). Such a nonmonotonic effect on MDH activity has been previously observed in response to phosphate ion (in the direction from oxalacetate to L-malate (*3*)). Mg^{2+} has been reported to have an activating effect on the reaction in blood serum (*4*). We can also note that high ionic strength has been reported to have a suppressive effect (*5*).

Materials and Methods

MDH Response. All experiments were performed with mitochondrial malate dehydrogenase from porcine heart, supplied as suspension in 70% saturated ammonium sulfate by ICN Biomedicals (Costa Mesa, CA). NAD^+ was used in the form of the free acid (purity 93−96%) and L-malate as crystalline free acid (purity 99%), both from ICN Biomedicals. MOPS was supplied as enzyme grade by Fisher Scientific (Pittsburgh, PA).

The absorption measurements of NADH were made in polysterene cuvettes with a 1-cm light path at 339 nm

* E-mail: biocomputing@cs.wayne.edu.

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with an Ultraspec II model 4050 spectrophotometer from LKB Biochrome (Cambridge, U.K.). The temperature in the dark chamber of the spectrophotometer was 29 °C at a room temperature of 23 °C. The spectrophotometer was controlled by a personal computer. The software for data acquisition was developed by us and polled for measurements at the highest rate supported by the spectrophotometer.

The assay protocol was derived from ref 6. The main modifications were the substitution of MOPS buffer for the potassium phosphate buffer to prevent precipitation with the Mg^{2+} and Ca^{2+} used as signal carriers (*7*) and a reduction of the substrate concentration.

Seven blocks of six assays each were run. All assays contained 2.3 mL of 0.12 M glycine/NaOH buffer at pH 10, 145 μL of 100 mM L-malate, and 145 μL of 25 mg/ml NAD^+ in 0.2 M MOPS at pH 6.9. In addition each assay contained 300 μL of a mixture of $MgCl_2$, $CaCl_2$, and water. For both $MgCl_2$ and $CaCl_2$, six factor levels (0 μL, 25 μL 2 M, 25 μL 4 M, 50 μL 4 M, 75 μL 4 M, 100 μL 4 M) were examined. To all 36 combinations water was added for a constant volume of 300 μL. The 36 assays were run in seven blocks of six cuvettes each. In the first cuvette of each block the assay with neither $MgCl_2$ nor $CaCl_2$ was repeated to control for block to block variation. The remaining 35 assays were distributed among the seven blocks. The assays were started by adding 110 μL of enzyme diluted in 0.1 M MOPS at pH 7.4 and inverting the sealed cuvette for mixing. The amount of enzyme was chosen to result in an absorbance increase that could conveniently be monitored.

Each cuvette was measured in intervals of ≈32 s, starting ≈2 min after initiation of the first of the six reactions in the block. The delay from the start of the reaction in the first cuvette until the first measurement of this cuvette was used to adjust the time of the reaction start for the first five cuvettes in each block.

For the MDH response surface shown in the results section (Figure 1), absorbance values at times between any two measurements were approximated by piecewise cubic polynomials (cubic spline interpolation). This was necessary, since for a given point in time usually some of the 36 assays did not have corresponding measurements at that point in time. The 36 interpolating functions were evaluated for a time 300 s after start of the reaction. To obtain the response surface (cf. refs 8 and 9), the absorbance values computed with these 36 functions were interpolated with cubic splines over the two varying factors ($MgCl_2$ and $CaCl_2$).

Signal Processor. The enzyme, chemicals, and spectrophotometer used for the XOR signal processing experiments were the same as described in the previous section. The enzyme solution used to initiate the reaction contained 5.4 mM NAD in 100 mM MOPS at pH 7.4 and 6 μL/mL enzyme suspension. The signal solutions for 0-signals and 1-signals both contained 7.1 mM L-malate in 114 mM glycine NaOH at pH 10.5. The solution for the 1-signal in addition contained 190 mM $MgCl_2$. The 0-signal was represented by the absence of $MgCl_2$.

The detailed operation of the signal processor will be described in the Results section. The components were connected with Luer fittings and tubing with an inner diameter of 1.6 mm. A flow cuvette was constructed by sealing five glass capillaries with silicon sealant into a 1 cm light path methacrylate cuvette (Fisher Scientific).

Results

Nonmonotonic Response of MDH to Mg^{2+} and Ca^{2+}. As noted in the Introduction our interest here is in the phenomenological response of an enzyme to milieu variations and not in the formulation or fitting of a mechanistic model. From this perspective MDH is a physical implementation of an input−output transform that maps milieu variables into catalytic activity. We take the NADH absorbance measured at a fixed time after the start of the reaction as the response, i.e., the output, of the system. The empirical relationship between factors in the milieu and the resulting output is characterized by a response surface.

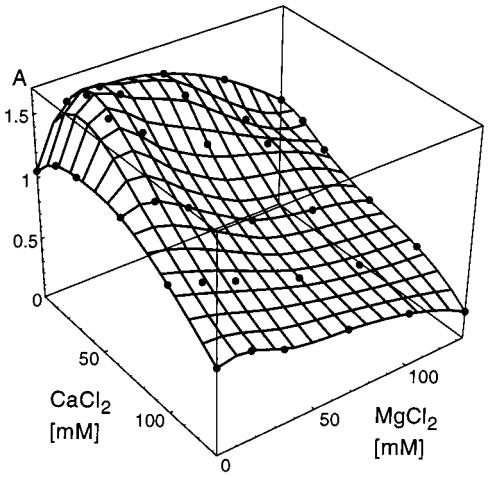

Figure 1. MDH response to $MgCl_2$ and $CaCl_2$. The convex shape of the surface means that the enzyme can differentiate signal patterns that are not linearly separable. The surface shows the absorbance values (*A*) at 300 s. Black dots indicate the concentrations at which measurements were taken. See Methods section for details.

Figure 1 shows the response of MDH with respect to changes in the concentration of Mg^{2+} and Ca^{2+}. The surface shows the NADH absorbance (at 339 nm) 300 s after start of the reaction. Note that the surface was obtained by interpolation and not by regression to an assumed model (see Methods section). The point of interest is the qualitative character of the response surface. As noted in the Introduction, the surface is convex (or strictly nonmonotonic) along both the $MgCl_2$ and $CaCl_2$ axes. The convex feature of the surface stays the same for a substantial span of the reaction course after 120 s, the time at which the earliest measurements were obtained.

Signal Coding and Pattern Classification. The response surface in Figure 1 can be interpreted from a computational point of view. The response of the enzyme to various milieu conditions in effect groups these conditions into classes set apart by level of catalytic activity. We view the enzyme as a physical realization of a pattern classifier (cf. ref 10). For a given protein the response is fixed. Some flexibility to implement different input-output transforms, however, is given by the freedom to choose the coding of the input signals and the interpretation of the response.

We will here consider a simple encoding scheme in which all signals are coded in such a way that no two signals share a signaling substance and each signal has a fixed encoding independent of the signal line on which it arrives. The latter condition allows for the implementation of commutative operations only, since no information regarding the order of the operands is available. The signals can be encoded by a fixed amount of one signaling substance or by a fixed mixture of substances.

In the simplest case only a single substance (or mixture) is employed. Each input line then provides one

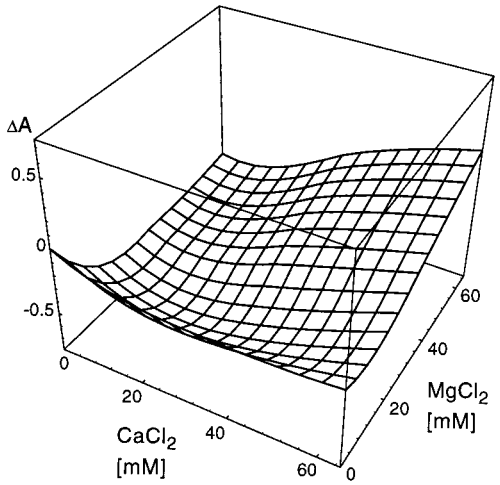

Figure 2. Difference of MDH response to distinct milieu conditions. The points on the surface correspond to the absorbance difference at a given $MgCl_2$ and $CaCl_2$ encoding of the 1-signal. Difference between 00 and a single 1-signal (i.e., 01 or 10). This corresponds to the difference in response to milieu state *a* and milieu state *b* in Table 1.

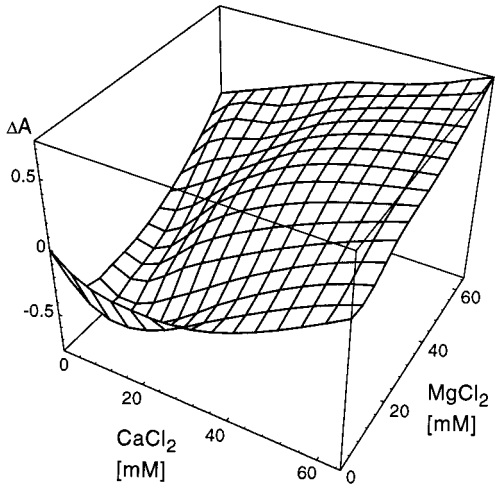

Figure 3. Difference in MDH response to 00 and 11 signal patterns, corresponding to the difference between milieu states *a* and *c*.

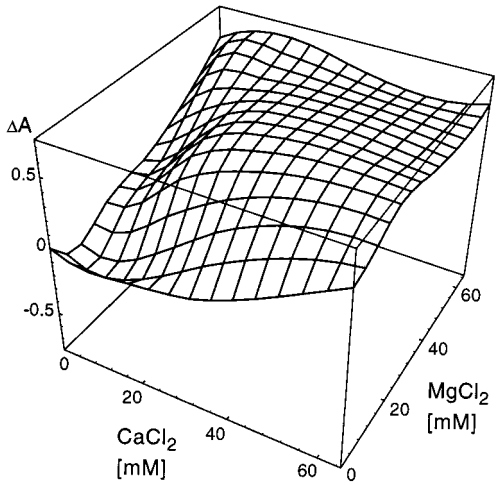

Figure 4. Difference in MDH response to the 11 signal pattern and 01/10 signal pattern, corresponding to the difference between milieu states *b* and *c*.

bit of information, i.e., can be in one of two states named 0 and 1. If two input lines are available the combination of the signals arriving at these lines can give rise to three milieu variations. These possible milieu states are here called *a* when both input bits are 0, *b* when one of the inputs is 0 and the other is 1, and *c* when both inputs are in the 1-state. By convention the 1-signal is represented by the presence of signaling substance and the 0-state by the absence of signaling substance.

Now we can consider how the input patterns are classified by different response levels. These will be denoted by $r(a)$ for milieu condition a, and similarly the response levels for the remaining two milieu states will be denoted by $r(b)$ and $r(c)$. The concentration of the substance (or substances) used to represent the 1-signal determines the response that the three milieu states (*a*, *b*, and *c*) give rise to and hence determines the possible separations into output classes. The difference in response for each pair of milieu states is shown in Figures 2−4. A change in the sign of the absorbance difference indicates a switch in relative strength of the responses produced by the two milieu conditions being compared.

The two bit input classifications considered here correspond to logic gates. The change in sign noted above distinguishes the concentration ranges that can be associated with different logic operations. Of the 16 possible operations with two bit input, 6 ignore one or all inputs (e.g., the output is constant) and 4 are not commutative and therefore cannot be implemented with the coding scheme considered here. The remaining 6 operations, which encompass common binary logic gates, are shown in Table 1. Each of the two input lines I_1 and I_2 can be in one of two states (0 or 1). The two input lines can give rise to the four possible input states shown in the top left of the table. The corresponding state of the output is shown below each of the four input states. Different ways of grouping the four input states (1−1, 0−1, 1−0, 0−0) into the two output states (0 and 1) are shown in each row of the lower part of the table. These correspond to the logic operations named on the left side. The letter "N" at the beginning of a name stands for "not-" and is equivalent to inverting the output state of the corresponding operation without the "N". Inverting the state of the input lines would result in an exchange of the rows for OR and NAND and the rows for NOR and AND.

Table 1. Input Classification for Common Logic Functions

line	input state							
I_1	1	0	1	0				
I_2	1	1	0	0				
operation	output state				*a*	*b*	*c*	signal strength, Δ*s*
AND	1	0	0	0	−	−	×	$r(c) - \mathrm{Max}(r(a), r(b))$
OR	1	1	1	0	−	×	×	$\mathrm{Min}(r(b), r(c)) - r(a)$
XOR	0	1	1	0	−	×	−	$r(b) - \mathrm{Max}(r(a), r(c))$
NAND	0	1	1	1	×	×	−	$\mathrm{Min}(r(a), r(b)) - r(c)$
NOR	0	0	0	1	×	−	−	$r(a) - \mathrm{Max}(r(b), r(c))$
NXOR	1	0	0	1	×	−	×	$\mathrm{Min}(r(a), r(c)) - r(b)$

The logic operations can also be expressed as classifications of the three milieu states, as shown in the columns *a*, *b*, and *c* of Table 1. A cross indicates that the milieu state should yield an active output, and a dash indicates that it should not. If the requisite conditions are satisfied for all milieu states in a given row the corresponding logic function can be realized. We here interpret a high response level as an active output and a low response level as inactive. However, the opposite convention could just as well be adopted and yield the same logic function if the signal strength formulas (last

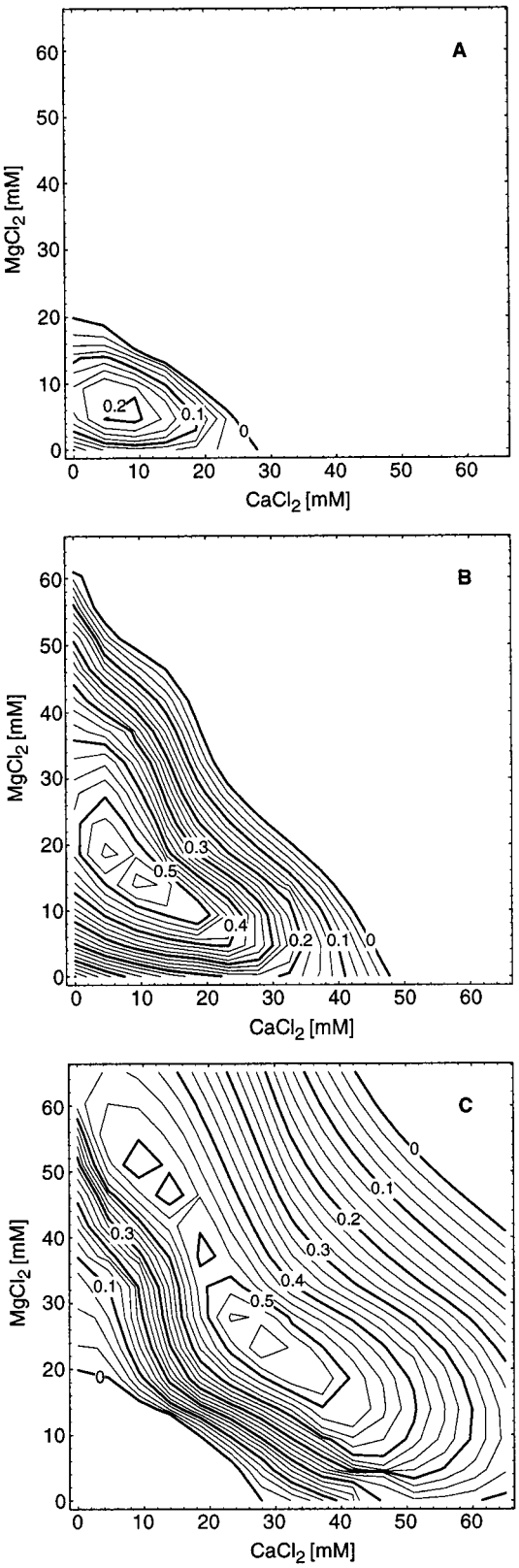

Figure 5. Ion concentrations corresponding to common logic operations. The contours show the signal strength, Δs, as specified in Table 1. Contours are shown only for positive signal strength. The area shown in (A) allows for implementation of AND, in (B) for the implementation of OR, and in (C) for the implementation of XOR.

column) for AND, OR, and XOR are exchanged with those for NAND, NOR, and NXOR, respectively. These formulas represent the minimum difference in the response between groups that need to be differentiated to implement a particular function. This minimum difference, Δs, represents the signal strength.

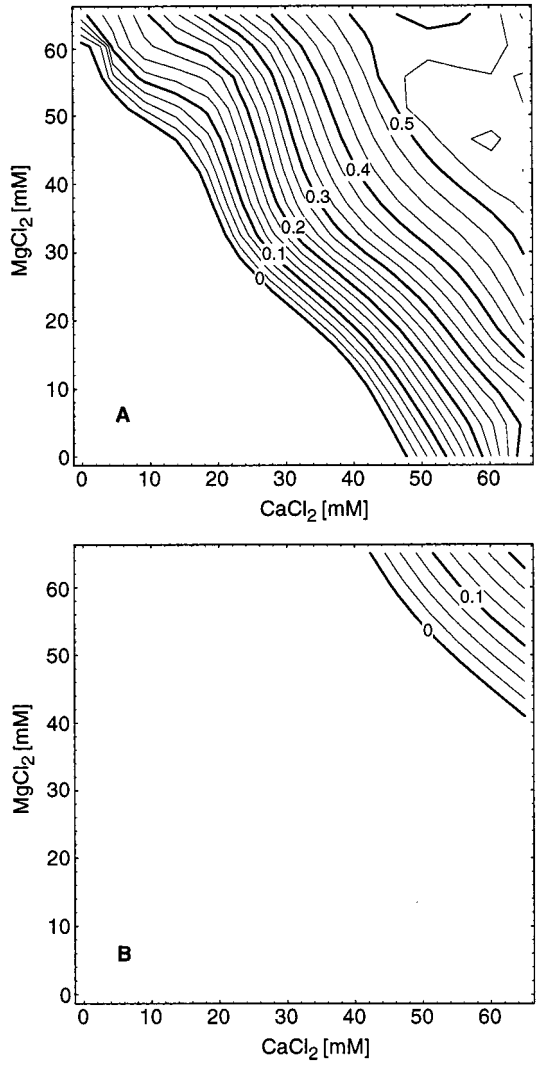

Figure 6. Negations of logic operations. The area shown in (A) allows for implementation of NAND and in (B) for the implementation of NOR.

The signal strength for an operation varies with the choice of concentrations used to encode the signals. If $\Delta s \leq 0$ for any choice of signal encodings, the particular operation cannot be implemented. In practice of course a high signal strength is desirable.

The experimental response surface (Figure 1) can be used in conjunction with the signal strength formulas in Table 1 to determine the ionic concentrations required to implement the various logic functions. The procedure is as follows. We first compute what the signal strength would be for the particular operation under consideration assuming that the 1-signal is encoded by a given $MgCl_2$ and $CaCl_2$ concentration. If the signal strength is positive the operation is implementable with the given encoding. If it is zero or negative the encoding cannot be used to implement the operation. This process is repeated at intervals over the entire range of concentrations. The range is one-half that of the response surface, due to the fact that an input of two 1-signals covers the range.

The contour diagrams depicted in Figures 5 and 6 represent the signal strength for five of the six operations in Table 1. The NXOR operation is not implementable with the convention that a high response is considered to be an active output.

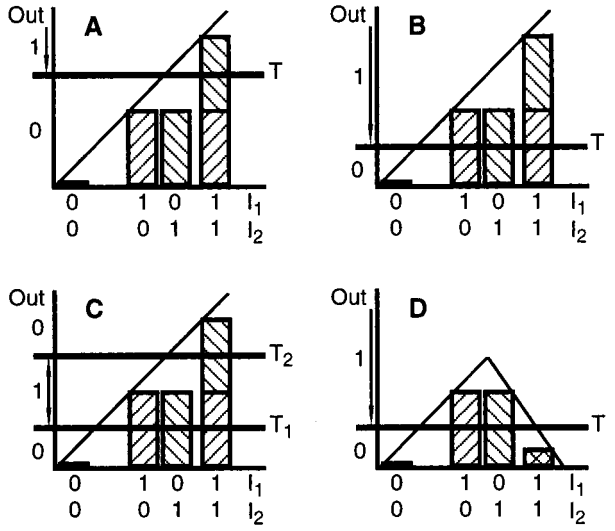

Figure 7. Linearly separable and inseparable input patterns. For linearly separable pattern groupings a monotonic response and a single threshold are sufficient for classification. For a monotonically increasing response a high threshold (A) results in an AND operation. A low threshold (B) converts this to an OR operation. For the linearly inseparable XOR two thresholds are required if the response is monotonic (C). A nonmonotonic element acts as a transform that allows the XOR to be realized with a single threshold (D).

The response classifies the patterns. To obtain a binary output it is necessary to threshold the response. Response levels below threshold are interpreted as a 0-output, and levels above threshold are interpreted as a 1-output. For signal encodings that have a positive signal strength for more than one classification the choice of threshold determines which logic function is implemented. Note that it is in some cases also possible to switch from one logic function to another without changing the threshold level. For example, the OR function can be implemented by coding the 1 signal by 20 mM Mg^{2+}. If 40 mM Ca^{2+} is added to the 1 signal, then the OR is converted into an XOR.

The XOR operation is linearly inseparable since the patterns to be placed in the 1 and 0 output categories cannot be differentiated by a single threshold (in contrast to NAND, AND, and OR operations). An element whose response is linear apart from a single threshold cannot perform the XOR, since it would be necessary for it to fire when the strength of the combined inputs exceeds a lower threshold and not fire when it exceeds a higher threshold. If the response of the element is nonlinear (strictly speaking nonmonotonic) then it is possible to eliminate the need for the higher threshold and therefore to convert the linearly inseparable pattern recognition problem to a linearly separable problem (Figure 7). An enzyme, to satisfy this requirement, must increase its activity in response to one concentration of the signaling substance but decrease it in response to a doubling of this concentration. The MDH response surface has the requisite nonmonotonic property and therefore encodings are available that allow the XOR operation to be implemented (as shown in Figure 5C). This means that the MDH dynamics serves to transform a linearly inseparable problem to a linearly separable problem. Alternatively stated, the enzyme groups the inputs so that it is possible to separate them with a single threshold.

Enzyme-Based Signal Processor. The response surface reveals the operations that are in principle possible. We have constructed a tabletop device to implement the operations and to facilitate exploratory experiments.

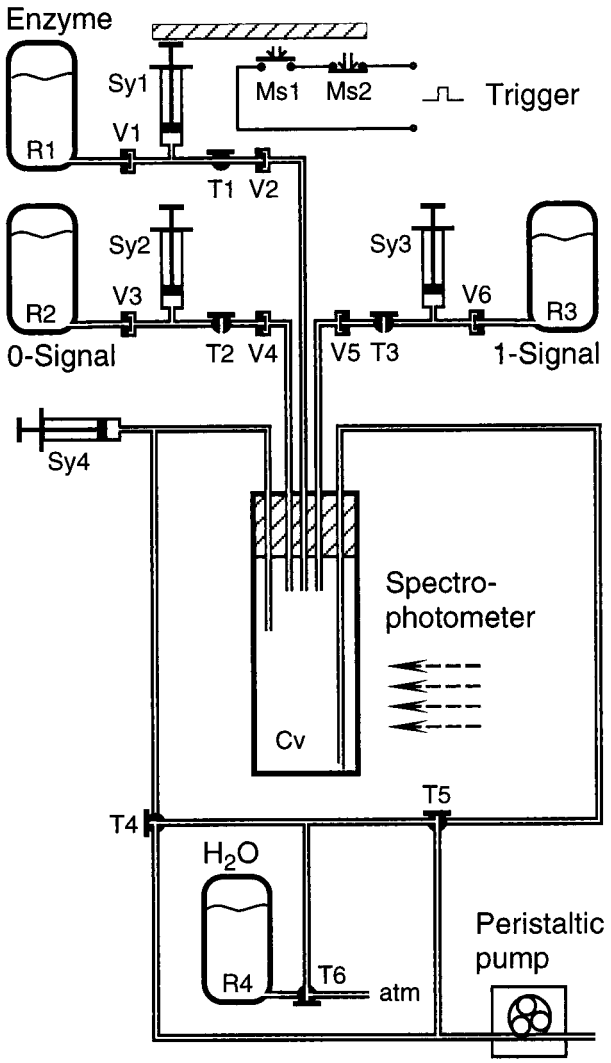

Figure 8. Schematic of setup used for implementing enzyme-based signal processing. See text for explanation.

The device is illustrated in Figure 8. At the core is a flow-cuvette (Cv) that serves as a mixing and reaction chamber. Five glass capillaries allow material to enter or be removed from the cuvette. Three of the capillaries are connected to small manual piston pumps, each comprising a disposable syringe, two check valves, and a T-valve (e.g., V1, Sy1, T1, V2). The T-valve serves during the cleaning cycle as a stopcock to prevent fluid from being drawn through the piston pump by underpressure in the flow cuvette. The remaining two capillaries are connected through T-valves T4 and T5 to a water reservoir (R4) and a peristaltic pump. These serve to flush the cuvette between consecutive processing cycles.

The three manual piston pumps (based on Sy1, Sy2, and Sy3) are connected to the reservoirs R1, R2, and R3. The syringe Sy1, connected to reservoir R1 that contains the enzyme, is coupled to two microswitches (Ms1 and Ms2). The latter provide a trigger signal when the enzyme is injected into the cuvette. Reservoir R2 and R3 contain the solutions that provide the 0- and 1-signals, respectively.

At the beginning of a signal processing cycle the flow cuvette (Cv) is empty. One of the four possible patterns of two 1-bit signals is injected into the cuvette. Each of

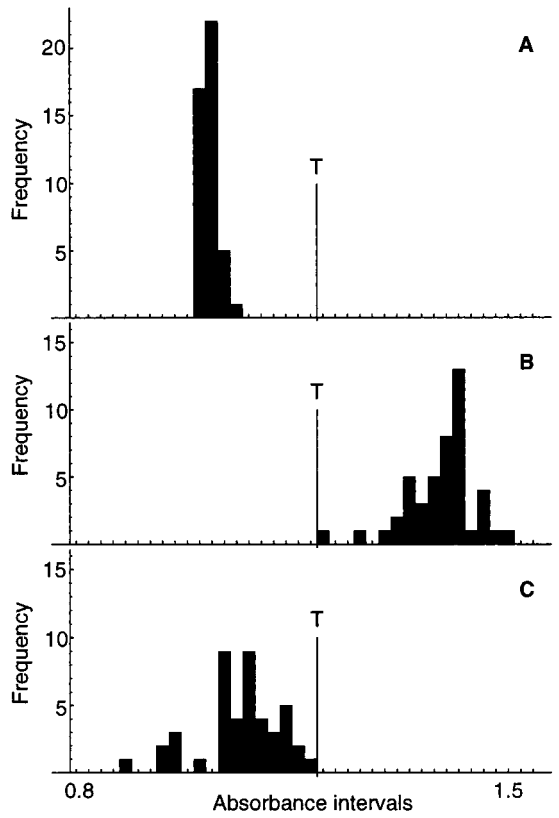

Figure 9. Distribution of MDH responses to two-bit input signal patterns for the XOR operation using the signal processing setup depicted in Figure 8. Bars represent absorbance intervals of 0.2. (A) Distribution for the 00 signal pattern for 45 presentations. (B) Distribution for 46 presentations of the 01/10 patterns. (C) Forty-four presentations for the 11 pattern. The order of presentation was mixed among the three cases. The threshold (*T*) can be used to obtain the correct classification.

the two signals is represented by 0.8 mL of signal solution. The corresponding air volume displaced from the cuvette is taken up by syringe Sy4. After the signal solutions have been injected into the cuvette, 0.5 mL of enzyme solution is injected to initiate the reaction. The injection of the enzyme solution through the capillary mixes the contents of the cuvette and also starts a timer (by means of Ms1 and Ms2) in the computer that controls the spectrophotometer. At a fixed time after the initiation of the reaction the computer queries the photometer for the current absorbance and uses this measurement as a classification of the signal pattern.

The processing cycle is followed by a cleaning cycle. Through appropriate settings of the T-valves T4, T5, and T6 the cuvette is first drained by the peristaltic pump, then flushed with distilled water and drained again. Syringe Sy4 is reset during the cleaning cycle.

From Figure 5C it can be seen that $MgCl_2$ and $CaCl_2$ can be used either alone or in combination to implement the XOR and also that the signal strength is stronger for $MgCl_2$ alone than for $CaCl_2$ alone. The bar graph in Figure 9 shows a series of 135 pattern presentations using $MgCl_2$ as the signaling substance. The number of patterns of each of the three types (00, 01 and 10, and 11) were nearly equal but presented in no particular order. The device separated all 135 patterns correctly, though in a few instances the signal strength was low. The measurements used to decide on output were made 10 s after the start of the reaction. This timing is close to what can be achieved with the spectrophotometer used and is not an inherent limitation. The signal strength recorded at earlier times was smaller but still sufficient to perform the classification, though in a less reliable manner. The processing speed is presumably limited by the turnover rate of the enzyme and by the detection limit, or alternatively by time required for the signals to affect the conformational dynamics of enzyme if, say, changes in fluorescence were taken as the response (*11*). The signal strength and hence reliability could always be increased by increasing the amount of enzyme.

Discussion

The reaction conditions in experiments reported here are outside of physiological range. No direct conclusions about the signal processing capabilities of enzymes in vivo are justified. The concentration of Mg^{2+} used to represent signals is probably higher than any reasonable magnitude change in the cell (*12*), and the pH at which the reaction was run is much higher than typical pH values in a cell (*13*). Nevertheless, the nonmonotonic response to the concentration of a single ion species suggests the possibility that enzymes are more than summing elements so far as their response to milieu conditions is concerned.

The effect of reaction conditions on MDH and possible kinetic mechanisms have been studied extensively (*14*−*19*). Ionic strength effects are discussed in ref 5. As noted earlier, high ionic strength has a suppressive effect on mitochondrial MDH under a variety of conditions, though exceptions have been noted. Ions not involved as reactants have in numerous cases been reported to enhance enzymatic activity (*20*). The nonmonotonic response to Mg^{2+} and Ca^{2+} is possibly due to opposition between the stimulatory and suppressive effects. Our purpose here, however, was not to investigate mechanism but rather to utilize phenomenology.

The connection between enzymes and logic operations has been considered by numerous authors, possibly starting with Sugita (*21*). The logic paradigm has in particular been used to model the kinetics of enzymes (*22*) and to analyze the control properties of metabolic networks (*2*, *23*). For molecular computing applications it seems unlikely that enzymes could compete with solid-state electronics in the domain of logic circuits. The point of the XOR demonstration is that enzymes can supply nonlinear input-output transforms, suggesting that complex networks of nonlinear base components could be implemented with biomolecules. Enzymes are ideal for this purpose, due to the fact that molecules can be precisely replicated, in contrast to the statistical aggregates used in solid-state technology. This means that their nonlinear characteristics can be precisely replicated. Networks built from base components with such characteristics could not function in a repeatable manner in the face of parameter variations in the components. The availability of precise nonlinear components would make it possible to implement given information processing functions with far fewer base components than equivalent logic networks would require. Conceivably the information processing capabilities of biological cells draw on this principle.

The restriction in the present experiments to two variable pattern recognition tasks means a restriction to common logic functions. Signal input patterns could be more complex, producing more complex milieu contexts. The advantage of conformational signal processing over linear processing elements could then become significant. We do not interpret the results presented here as implying that enzymes in biological cells act as logic gates

or that technological applications could provide any advantage over electronic technologies for the performance of such simple pattern processing tasks. The enzyme is more appropriately viewed as a context-selective entity that uses its conformational dynamics to fuse milieu influences in a way that modulates action. The XOR result is indicative of the possibility that enzymes can in effect transform milieu pattern processing tasks that are difficult because of their inherent context sensitivity into output behaviors that partition these tasks in useful ways. Enzyme species could be utilized in combination to achieve response surfaces with computationally rich potentialities that could not easily be deduced from the response surface of the base components. Developing response surfaces for more complex signal patterns will be necessary in order to determine the utility of this approach.

Acknowledgment

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