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Implementation of a multifunctional logic gate based on folding/unfolding transitions of a protein

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Abstract

Currently there is great interest in the development of (bio)chemical devices that can act as logic gates at the molecular level. In this work we show that the protein cytochrome *c* (cyt *c*) can be used to perform a variety of logic operations such as YES, NOT, AND, OR, and XOR. The protein accepts chemical input signals in the form of denaturants that can induce unfolding of the polypeptide chain. These conformational changes lead to alterations of the protein fluorescence intensity which is used as the output signal. The specific type of logic function performed by the protein depends on the types of denaturants and on the solvent conditions used in the experiment. We describe a dialysis cell that can be regarded as a simple hardware version of a protein gate. This device allows reversible switching of the input signals. The operation of this cell is based on a simplified strategy that employs just one type of chemical input signal, namely an aqueous solution of hydrochloric acid. The logic function performed by the device is determined by a "gate controller" (GC) input.

Keywords: Boolean logic; Protein folding; Molecular computing; Fluorescence

1. Introduction

Molecular computing is a research area that aims at developing alternatives to traditional silicon-based information processing systems [1,2]. It is an intriguing idea that molecules, instead of electronic devices, could be used for computational functions. This approach could eventually lead to smaller, faster and more energy-efficient devices; it could also open up completely new strategies for tackling difficult computational tasks. The area of molecular computing is still in a very early stage of development. At present it is not clear whether any of the numerous developments in this area has the potential to seriously rival the role of semiconductor electronics in information technology. However, a number of approaches show great promise and they could turn out to be stepping stones for molecular-based information technologies [2]. DNA computing [3–6] and similar methods based on RNA [7] have been used to solve NP-complete problems. Other researchers have suggested the use of biomembranes [8] or biochemical reaction networks [9–11] for computational applications. Photosynthetic reaction centers [12], supramolecular organic complexes [13,14], and carbon nanotubes [15] could be used as components of molecular electronic devices. Bacteriorhodopsin has been employed to implement optical data storage media [16–18].

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Table 1
Truth tables of some logic operations

Operation	Input 1	Input 2	Output
YES	1		1
	0		0
NOT	1		0
	0		1
AND	0	0	0
	1	0	0
	0	1	0
	1	1	1
OR	0	0	0
	1	0	1
	0	1	1
	1	1	1
XOR	0	0	0
	1	0	1
	0	1	1
	1	1	0

Computer processors and other electronic devices that are currently in use are based on simple Boolean operations performed by logic gates [19]. Logic gates receive one or more binary (0, 1) input signals and produce a single binary output signal. The output depends on the input signal(s) and on the logic function performed by the gate. Logic functions are commonly represented in truth tables, some examples of which are shown in Table 1. In electronics, the binary values of 1 and 0 are represented by high and low voltage levels, respectively. First attempts towards the development of electronic molecular gates have been described recently [20,21]. However, molecular logic does not necessarily have to be based on electronics. In principle, the input and output signals of a gate can have any form, for example, they could be mechanical, chemical, or optical. All-optical molecular logic gates have been implemented by using thin films of bacteriorhodopsin [22,23]. A number of synthetic organic complexes accept chemical input signals and produce a fluorescence output that can be interpreted as the result of a logic operation. These molecules can be classified as "chemophotonic" logic gates. For example, an anthracene derivative has been described that shows a high fluorescence intensity (output) only if sodium ions (input 1) and protons (input 2) are present in solution. Therefore the output of this gate is always 0, except when both inputs are 1, corresponding to the logic function AND [24]. Molecular gates of this kind can perform simple arithmetic operations [25]. Numerous other switchable molecules have been described that can be used for logic operations or related applications [26–36].

Just like their traditional electronic counterparts, the chemophotonic molecular gates mentioned so far are only designed to perform one single logic operation. It would be a significant advance if molecular devices could be implemented that are capable of performing multiple logic functions. This could greatly simplify the design and the versatility of future integrated devices that use molecular logic. The issue of multifunctionality is addressed in the current study where we explore the use of a biological molecule as a chemophotonic logic gate. The heme protein cytochrome *c* (cyt *c*, MW 12.4 kDa, see Fig. 1) has a single tryptophan residue (Trp59). In the native protein, Trp59 is in close proximity to the heme group which leads to efficient intramolecular quenching of the Trp fluorescence [37–40]. Like many other proteins, cyt *c* can be reversibly unfolded by the addition of various denaturants such as acid, base, or urea [38,41,42]. Partial or complete unfolding increases the Trp–heme distance, therefore disrupting energy transfer between the two chromophores [37,40]. As a result, unfolded cyt *c* shows an intense Trp fluorescence, whereas the

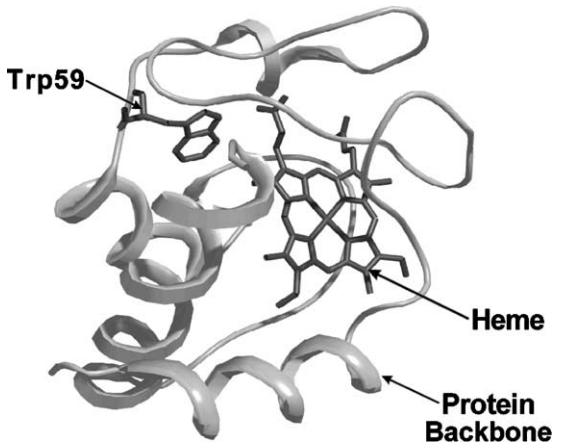

Fig. 1. Schematic diagram showing the native structure of the protein cyt *c*. Note the close proximity of the single tryptophan (Trp59) to the heme group that leads to efficient fluorescence quenching.

native protein is virtually non-fluorescent. The current study is divided in two parts: first we describe a set of experiments as proof-of-principle that folding/unfolding transitions of cyt *c* can be interpreted as logic operations. In the second part we introduce a novel concept that uses a greatly simplified strategy and involves an additional control input to determine the logic function performed by the protein. A setup is described that allows reversible switching of the input signals and that can be regarded as a hardware model of a protein gate.

2. Methods

Horse heart cyt *c* was obtained from Sigma (Oakville, Ont.) and was used without further purification. Also tris-(hydroxymethyl) aminomethane, tryptophan, NaH_2PO_4 and Na_2HPO_4 were obtained from Sigma. Sodium hydroxide was purchased from BDH (Toronto, Ont.), hydrochloric acid from Caledon (Georgetown, Ont.), methanol from EM Science (Gibbstown, NJ) and urea from Boehringer Mannheim (Montreal, Que.). Fluorescence emission spectra of 5 μM cyt *c* were recorded at room temperature on a Spex Fluorolog 3 fluorimeter (Instruments S.A., Edison, NJ) at an excitation wavelength of 280 nm. A spectral bandwidth of 5 nm was used for excitation and emission. The intensities of the fluorescence spectra reported in this work have been corrected for volume changes.

3. Proof-of-principle: cyt *c* used as a chemophotonic logic gate

We will first describe the results of some simple experiments to demonstrate that cyt *c* can be used as a chemophotonic logic gate, similar to gates that are based on synthetic organic complexes [24–26,31–33,35,36]. Cyt *c* accepts input signals in the form of denaturants that can induce unfolding of the protein. Different denaturants correspond to different input channels. Each input signal can have a value of 1 or 0, corresponding to the presence or absence, respectively, of a certain denaturant. The fluorescence intensity of the protein is interpreted as the output of the gate and it is reported relative to that of free Trp in water. The output is considered to be 0 when the intensity of the spectrum peaks below 0.04, whereas an output above 0.08 is interpreted as 1. Any output that falls between these two values would correspond to an invalid result of a logic operation. It will be seen that this choice of thresholds allows for an unambiguous differentiation between 0 and 1. The concept of using two threshold levels to define a "forbidden range" is commonly employed for conventional electronic gates [19].

Fig. 2A shows the use of cyt *c* in aqueous solution as an AND gate. The binary value of input 1 corresponds to the presence (1) or absence (0) of 4.8 M

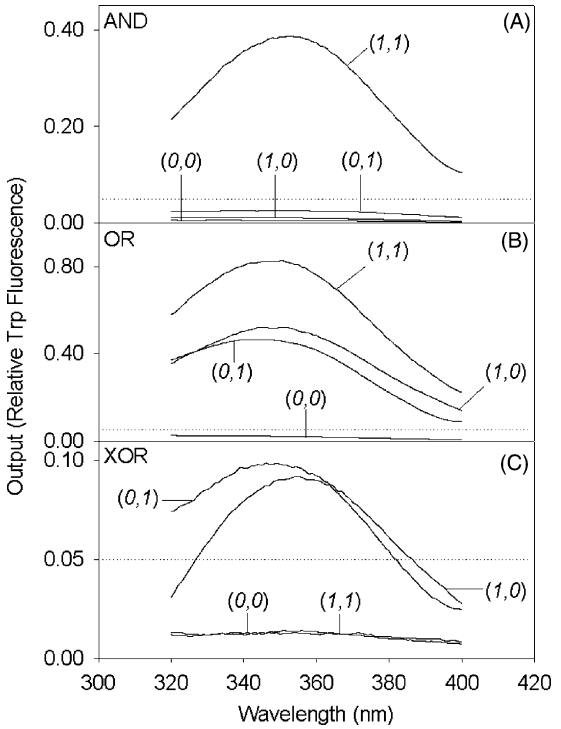

Fig. 2. Fluorescence emission spectra demonstrating the use of cyt *c* as a multifunctional logic gate. The four spectra depicted in each panel show the response of the protein to the presence or absence of two different denaturants: (1, 1) both denaturants are present; (1, 0) only the first denaturant is present; (0, 1) only the second denaturant is present; (0, 0) both denaturants are absent. Any signals below a relative fluorescence intensity of 0.04 (lower dotted line) represents an output of 0, signals above 0.08 (upper dotted line) represents an output of 1. (A) AND gate. Denaturants: 4.8 M urea and 1.25 mM HCl; solvent: water; (B) OR gate. Denaturants: 7.1 M urea and 1 mM HCl; solvent: water/methanol (50:50 v/v); (C) XOR gate. Denaturants: 0.1 M NaOH and 0.1 M HCl; solvent: 50 mM Tris in water.

urea. Likewise, input 2 represents the presence (1) or absence (0) of 1.25 mM hydrochloric acid. For this experiment, the denaturant concentrations were chosen relatively low, so that the presence of either denaturant alone does not induce unfolding of the protein. Therefore, in the presence of only one denaturant, there is a low fluorescence intensity, corresponding to an output of 0. When both denaturants are present together, the protein unfolds and an intense fluorescence signal is observed. Thus, an output of 1 occurs only when both inputs are 1.

Acid and urea can also be used to operate cyt *c* as an OR gate (Fig. 2B). For this purpose, the protein is dissolved in a 50:50 (v/v) mixture of methanol and water. In the absence of any other denaturants, the presence of methanol does not lead to an increased fluorescence intensity. Upon addition of either 7.1 M urea or 1 mM HCl, the protein unfolds and a high fluorescence intensity is observed. Also the presence of both denaturants leads to a high fluorescence intensity. Thus, when either one or both of the inputs is 1, the output of the gate is 1. The cosolvent methanol is used for this OR gate because in the absence of methanol, urea-induced denaturation causes only a relatively small increase in fluorescence. It is known that methanol leads to a destabilization of the native cyt *c* structure, making it more susceptible to unfolding by relatively "weak" denaturants such as urea [40,43].

In order to use cyt *c* as an XOR gate, acid and base were chosen as denaturants (Fig. 2C). This experiment was carried out in 50 mM Tris buffer. The addition of either 0.1 M HCl or 0.1 M sodium hydroxide (NaOH) causes a strong fluorescence signal. When both denaturants are present in equimolar amounts, a neutral solution is obtained. Under these conditions, the protein refolds and fluorescence is lost. Therefore, the output of the gate is 1 only if one input is 1 and the other is 0. The use of a buffer system for the XOR gate ensures that neutralization occurs after addition of both acid and base, even if there are small discrepancies in the concentrations of the two denaturants.

It is known that in aqueous solutions containing 0.1 M HCl, cyt *c* does not adopt a fully unfolded conformation. This is because the intramolecular charge repulsion of the fully protonated polypeptide chain is partly compensated by the high concentration of chloride anions in solution [44]. Most likely, this is the cause for the lower fluorescence intensity of the denatured protein in 0.1 M HCl compared to the unfolded protein in the case of the AND and OR gates. Cyt *c* also shows a lower fluorescence intensity in 0.1 M NaOH than under the denaturing conditions used for the two previous logic gates. Circular dichroism and absorption spectra indicate that cyt *c* retains a certain degree of secondary and tertiary structure in 0.1 M NaOH (data not shown). Therefore, the relatively low fluorescence intensity observed under these basic conditions is likely due to incomplete unfolding.

In addition to the logic gates described above, the single-input YES and NOT functions can be implemented. YES logic requires that the output be the same as the input, and NOT logic requires the output to be opposite to the input. Fig. 2B shows that an input of acid (1), causes an output of fluorescence (1), whereas in the absence of acid (0), there is no fluorescence (0), corresponding to YES logic. NOT logic can be accomplished by using cyt *c* in basic solution as the input 0 state; this will cause the output to be 1. The addition of acid corresponds to an input of 1, which results in an output of 0 (see Fig. 2C).

These data demonstrate a surprisingly simple concept that allows the interpretation of cyt *c* folding and unfolding transitions as logic operations at the molecular level. The individual functions performed by the protein are analogous to those of synthetic chemophotonic gates [24–26,31–33,35,36].

4. Hardware implementation of a protein gate: using gate controllers (GCs) and a novel type of input scheme

In order to develop a more practical concept of cyt *c* as a molecular logic device we implemented a simple hardware model of a protein gate. A solution of 10 μM cyt *c* was "immobilized" between the walls of a fluorescence cuvette and a dialysis membrane. Such a setup allows reversible switching of the gate, i.e. denaturants can be introduced into the protein solution and they can be removed by dialysis. Classical electronics uses just one type of input signal, i.e. all input values of 0 and 1 correspond to low and high voltage levels, respectively. The strategy used in the last section is more complex because different logic operations require different types of chemical input signals. We now present a modified strategy that allows the use

of just one type of chemical input signal, namely an acidic solution of hydrochloric acid, 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 at pH 1.5. To maintain the multifunctionality of the gate a GC is employed that determines the specific logic function performed by the protein. The GC is an additional input; it is comparable to the bias signal applied to neurons in artificial neural networks [45]. It will be seen that switching between different logic functions can be achieved by simply changing the type of GC solution.

A schematic diagram of a two-input protein gate is shown in Fig. 3. A loop of 5 cm dialysis tubing (molecular weight cut-off 8 kDa) is submerged in protein solution inside a fluorescence cuvette. Solution is pumped through this loop at a flow rate of 12 ml/min while the protein outside of the bag is stirred continuously. The composition of the solution entering the dialysis bag is variable, depending on the gate inputs and on the type of GC solution used. The binary input values of the gate are determined by two valves that control the liquid flow from two-acid reservoirs. An input of 0 corresponds to a closed valve, and an input of 1 is represented by an open valve. When both

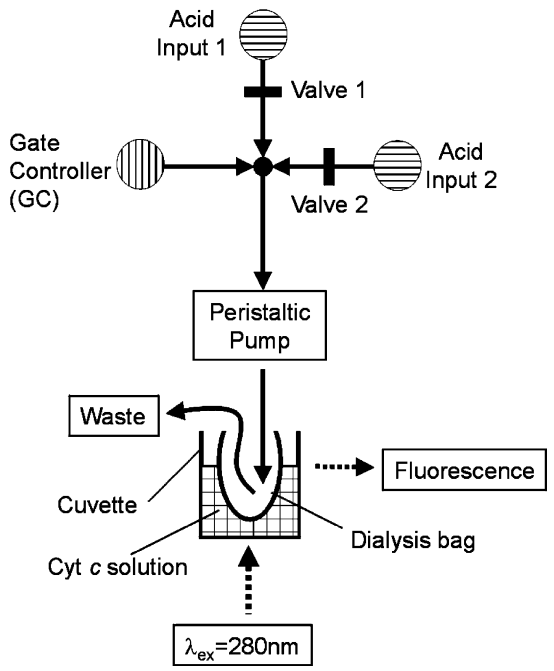

Fig. 3. Schematic diagram of the two-input protein gate used for this work. For details see text.

control valves are closed (input (0, 0)) the solution entering the dialysis bag consists entirely of GC. If one valve is open (input (0, 1) or (1, 0)) the solution will consist of a 1:1 mixture of GC and acid input; if both valves are open (input (1, 1)) the solution will consist of a 1:2 mixture of GC and acid input. The fluorescence intensity of the protein solution, integrated for $\lambda > 325$ nm, is monitored continuously by a photomultiplier with a cut-on filter mounted in front of it. This signal represents the output of the gate. The output is considered to be 0 when it is lower than a relative Trp fluorescence of 0.23 and it is interpreted as 1 when it is higher than 0.33.

One of the simplest logic functions that can be performed by the protein gate is YES. For this function a GC consisting of 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 at pH 6.9 is used. For this single-input gate only one input valve is switched while the other one is permanently closed. Fig. 4 shows that a chemical input of 1 results in a fluorescence output of 1; an input of 0 results in an output of 0. Switching was fully reversible during the three cycles studied in the experiment. When two-input channels are used with this type of GC, the gate performs the OR logic function (Fig. 5). The output of the gate is 0 if both inputs are 0, in all other cases the output is 1. When an additional input is added, the gate performs the three-input OR function (Fig. 6). In this case the output is 0 only if all three inputs are 0. The two-input function AND can be performed by using a GC consisting of NaOH in water at pH 12.4 (Fig. 7). This GC compensates one acid input but it does not compensate the effects of two-acid inputs. Therefore the output of the gate is 1 only if both input signals are 1. It is 0 for all other combinations of input signals. Fig. 8 shows the results of an experiment where the protein gate is configured to perform the single-input logic function NOT. The GC for this gate consists of an aqueous solution of NaOH containing 2 M urea at pH 13. Under these relatively harsh conditions the protein is unfolded when it is dissolved in pure GC, hence it shows a fluorescence output of 1 for an input of 0. An acid input of 1 neutralizes a substantial portion of hydroxide ions in solution and induces the protein to refold, resulting in an output of 0. Unfortunately, the switching behavior of this gate is not fully reversible when subsequent switching cycles are studied. While the protein continues to responds to changes of the input signal, the amplitude of

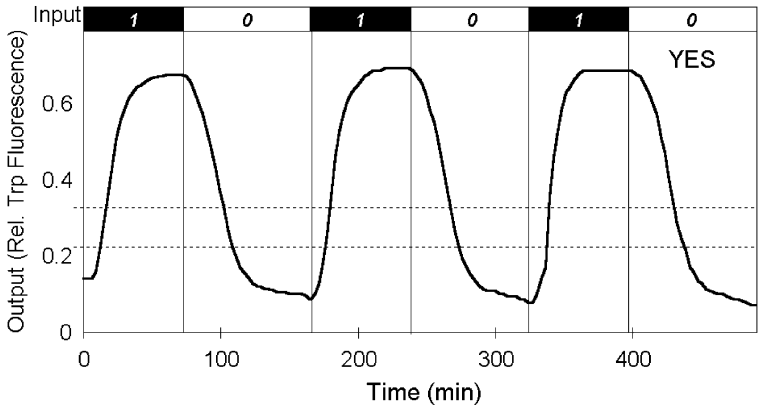

Fig. 4. Protein gate used for the YES logic operation. The total Trp fluorescence (integrated for $\lambda > 325$ nm) is shown as a function of time while switching the input signal through three on/off cycles. The input signal is indicated in the top portion of the diagram. A fluorescence output below a relative Trp fluorescence of 0.23 (lower dashed line) is interpreted as 0; an output above 0.33 (upper dashed line) is interpreted as 1. The GC in this case is an aqueous solution of 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 at pH 6.9.

the fluorescence signal decreases gradually. A similar, yet less pronounced effect was observed for the AND gate (Fig. 7) when it was switched through a second cycle (data not shown). Hydrolysis of cyt *c* with subsequent loss of protein fragments through the dialysis membrane could be one possible explanation for this effect. However, hydrolysis reactions of proteins usually require much harsher conditions such as higher base concentration and elevated temperature [46–48]. Attempts to detect proteolytic protein fragments by electrospray mass spectrometry after extended protein incubation under the conditions used for the NOT and AND experiments showed negative results (data not shown). Another possibility is that the conditions used in these experiments promote irreversible protein aggregation. This explanation seems unlikely since aggregated cyt *c* would be expected to show an elevated fluorescence level, similar to the unfolded protein in solution. A more likely explanation for the loss of signal intensity could be a slow degradation of the dialysis membrane under the alkaline conditions used in these experiments. If this were the case, the decreasing signal amplitude could be attributed to the loss of protein through the membrane. Other studies

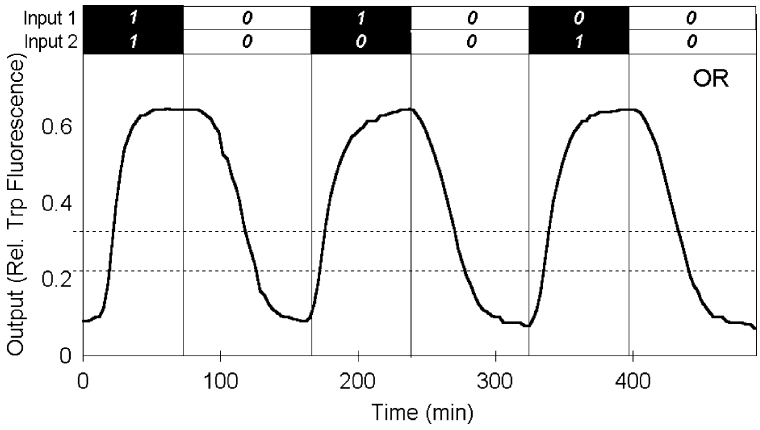

Fig. 5. Protein gate used for the OR logic operation. The notation is the same as in Fig. 4, except that now the values of two-input signal are depicted in the top part of the diagram. The GC used was the same as for the YES logic gate.

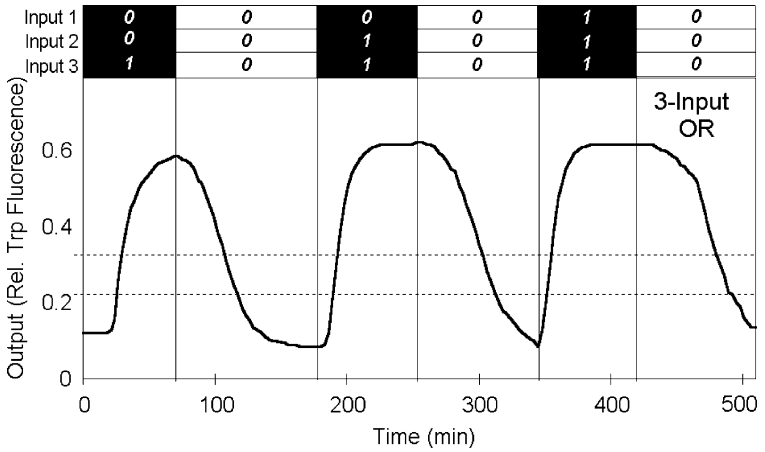

Fig. 6. Protein gate used for the three-input OR logic operation. The notation is analogous to that used for Figs. 3 and 4. The GC used was the same as for the YES logic gate.

on molecular gates have completely avoided the issue of switching reversibility or they have described gates that can only be switched once [20]. Therefore we do not regard the observed signal degradation as a significant problem at this time. It should be possible to improve this aspect of the gate by using proteins that are chemically immobilized on surfaces instead of trapping them in a dialysis cell [49–52].

The data shown in Figs. 4–8 demonstrate the response of the gate for different input signals while keeping the GC input constant. For the experiment depicted in Fig. 9 the opposite strategy was used. The protein gate was operated with one acid input opened (1) and the other acid input closed (0) while the gate function was switched by changing the type of GC. Exposure of the gate to OR GC results in an output of 1, switching to AND GC induces an output of 0. The gate output returns to 1 when the protein is exposed to YES GC, exposure to NOT GC finally results in an output of 0. This behavior clearly illustrates the multifunctionality of the protein gate and it demonstrates that there are two complimentary ways to switch the gate by either changing the input signal(s) or the type of GC.

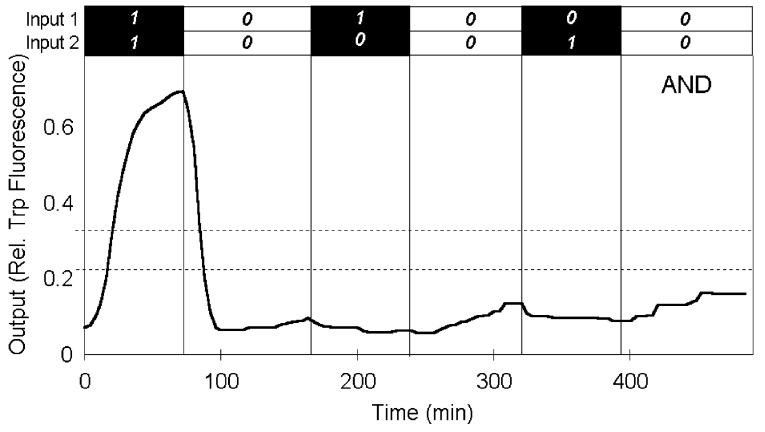

Fig. 7. Protein gate used for the AND logic operation. For notation see the captions of previous figures. The GC used was an aqueous solution of NaOH at pH 12.4.

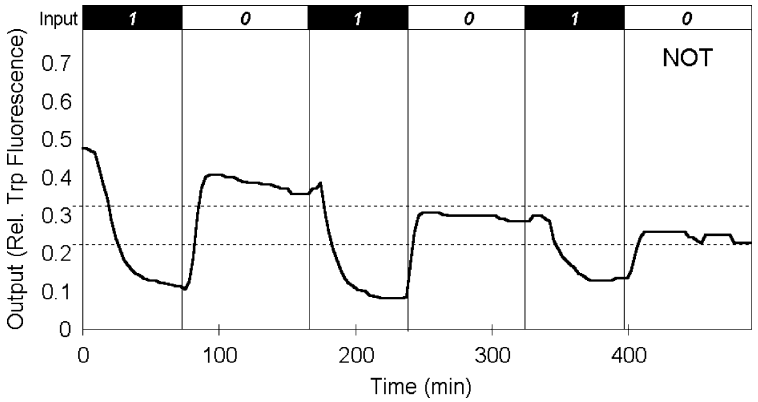

Fig. 8. Protein gate used for the NOT logic operation. For notation see the captions of previous figures. The GC used was an aqueous solution of NaOH and 2 M urea at pH 13.

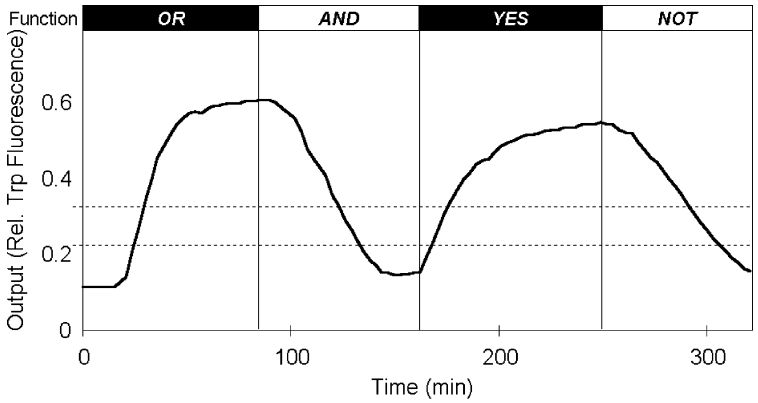

Fig. 9. Response of the protein gate to different GC signals while keeping one input valve open and the other input valve closed. For details see text.

5. Strengths, limitations, and possible applications of protein gates

This work clearly demonstrates the feasibility of chemophotonic logic gates that are based on protein folding reactions. The chemical input signals of most synthetic chemophotonic gates act through discrete and stoichiometric recognition events. In contrast, input signal recognition in the case of cyt *c* is based on non-stoichiometric interactions [35]. Cyt *c* acts as a multifunctional gate, i.e. it can perform more than one logic function and it can even perform functions requiring different numbers of input signals. Conventional electronic gates are hard-wired and therefore any of these devices is only capable of performing one specific logic function. At the molecular level, multifunctionality has previously been demonstrated for optical gates [22,23] and to a certain degree, for electronic gates [20] but never for chemophotonic gates. In the first part of this work we demonstrated that different logic functions can be performed by using chemically distinct input signals. Using this concept, relatively large variations in the output intensities of the different gates were observed (see Fig. 2). It is more elegant and conceptually simpler to use a modified concept that employs only

one type of input signal (acid) that allows control of the type of logic function by a GC input. This novel strategy results in a more consistent output intensity. For all the different gates studied an output of 1 is represented by relative fluorescence intensities between 0.5 and 0.7. It appears that certain functions such as XOR and INH [31] can only be implemented by using chemically distinct inputs. However, in order to achieve computational completeness it is not necessary to have all possible types of logic gates available. The operations AND, OR, and NOT (all of which can be performed using the modified concept) form a complete set, i.e. in principle any digital logic circuit can be created from these gates [19].

The coupling of molecular gates is a key problem that has to be solved before these devices can be put to use in integrated data processing systems. For conventional electronic gates coupling is straightforward since these devices are "macroscopic" objects and the output of one gate can directly serve as input signal for another gate. So far it has not been possible to couple logic gates at the molecular level. Most studies on chemical logic gates do not address this issue. In this paragraph we will briefly speculate on possible ways to integrate protein gates into larger systems. One difficulty encountered for chemophotonic gates is the different nature of the input and output signals. However, this in itself is not an insurmountable problem; data processing in biological systems is based on neurons which receive electrical input signals and produce chemical output signals. An obvious, but maybe not a very practical way to implement a protein-based data processing system would be to monitor the output of individual gates optically and to use these signals to control the flow of denaturant inputs and GC signals to other gates. Changes in the fluorescence output of a protein gate are a manifestation of major conformational changes. In order to couple protein-based logic gates it might be a better approach to exploit these conformational changes more directly, for example in the context of switchable pores that regulate the flow of chemicals in a microfluidic system. Another possibility involves the use of enzyme cascades where the product of one enzymatic reaction affects the conformation (and therefore the activity) of other enzymes, thus merging the concepts of protein gates and biochemical reaction networks [9–11]. One could also think of electronic systems consisting of nanowires [13] that are connected by molecular switches which are operated by conformational changes of protein molecules. In all these scenarios one type of protein could perform a wide range of logic functions, as shown in this study. Protein gates could be useful in conjunction with biosensors or other "wet" devices to perform a certain degree of data processing at a non-electronic level. It might also be interesting to explore the use of protein folding/unfolding transitions for molecular machines, a research area that is somewhat related to molecular computing [26,53,54].

An interesting issue is the time required to switch a protein gate. Protein folding reactions can occur on the microsecond time scale [55]. In principle, it should therefore be possible to implement protein-based data processing systems that operate at a clock speed of some MHz. This is still relatively slow compared to state-of-the-art microelectronics. However, it seems possible that the slower switching time of protein gates could be more than compensated by their multifunctionality. An electronic gate may be fast, but it can perform only one single function. In contrast, the function of a protein gate can be adapted to the requirements of the specific task at hand. The dialysis-based setup used for this work has an extremely slow response time because solvent exchange involves molecular diffusion through a porous membrane. Faster response times could be achieved by using chemically immobilized proteins in direct contact with the solvent [49–52]. As mentioned above, this approach would also improve the long-term stability of the gate. Research projects in this direction are currently under way in our laboratory.

In conclusion, this work shows that proteins like cyt *c* can be regarded as an interesting alternative to other molecular logic gates that have been described recently. We hope that future work in the fascinating area of molecular computing will lead to the development of (bio)chemical devices that can be applied to real-world problems and that will complement existing silicon-based technologies.

Acknowledgements

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