pH-induced bistable dynamic behaviour in the reaction catalysed by glucose-6-phosphate dehydrogenase and conformational hysteresis of the enzyme

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1. Bistable (multiple stationary states) dynamic behaviour in the activity of glucose-6-phosphate dehydrogenase that was subjected to successive pH change was demonstrated in an open continuously stirred tank reactor. Although the enzyme under study did not exhibit an autocatalytic effect and was homogeneously distributed, bistability was shown to occur. 2. The successive pH changes of the enzyme solution corresponded to a pH transition ($8.3 \rightleftharpoons 2$), i.e. an acidification (forward direction) and an alkalization (reverse direction). By use of intrinsic protein fluorescence methods, a glucose-6-phosphate dehydrogenase conformational hysteresis was shown to exist concomitant with the pH transition before and after enzyme injection into the reactor. 3. The results obtained suggest that the enzyme behaves, conformationally, as a memory device that stores information about its pH history (i.e. the enzyme records information in its structure about the environment to which it was previously exposed) and transduces it in a non-linear dynamic fashion, producing the bistable behaviour observed in the open reactor.

INTRODUCTION

Conformational changes in proteins have been shown to play a general role in enzymic catalysis. Several results have confirmed the general idea concerning the determining role of conformational phenomena in enzymic activity [1–4].

A large number of enzymes exhibit slow transitions or conformational changes that have been shown to affect the overall reaction rate, thereby producing hysteretic behaviour [5,6]. These slow conformational changes, which may generate non-hyperbolic kinetics, have been shown to occur upon desorption of the last product from the active site [7].

The concepts mentioned above represent different viewpoints of the same basic process, defined as hysteretic behaviour, which results from slow transitions [6,8–12]. In order to avoid confusion, and principally since the term ‘hysteresis’ has been employed for designating transient time-dependent slow conformational changes in enzymes, the word ‘bistability’ is used in the present paper. Bistability (also called multiple steady states) refers to the dynamic behaviour of an open system, i.e. the occurrence of two possible steady states for the same set of control parameters. The occurrence of one or the other of the steady states depends only on the initial conditions, i.e. on the system history [13]. The existence of multiple steady states requires the coupling of at least two processes, one of them exhibiting non-linear behaviour with respect to a critical parameter. The term ‘hysteresis’ is applied throughout this work to the glucose-6-phosphate dehydrogenase conformational behaviours. The word ‘conformation’ means in this case the state of the protein corresponding to a fixed pattern of secondary bonds [1].

Bistable behaviour in the activity of immobilized papain and uricase [14] and urease [15] as a function of pH has been demonstrated, and the observed dynamic behaviour has been attributed to the coupling of an autocatalytic reaction and diffusion. Neumann [16] has demonstrated experimentally the existence of molecular hysteresis in RNA as a function of pH. Long-lived hysteresis had been attributed to thermodynamically metastable states and co-operative non-equilibrium transitions [16,17].

Our results show that, in an open system, an enzyme with Michaelis–Menten behaviour, glucose-6-phosphate dehydrogenase [18–20], when subjected to a pH history, imposes a bistable dynamic behaviour on the system. The enzyme behaves, conformationally, as a memory device storing information about its pH history, i.e. the enzyme records information in its structure about the environment to which it was previously exposed. The main result of the present paper is that hysteretic behaviour in the conformation of the enzyme can produce bistable dynamic behaviour in an open homogeneous reactor.

EXPERIMENTAL

Materials

Glucose-6-phosphate dehydrogenase (type XV) and hexokinase (310 units/mg of solid; type F-300) were from baker’s yeast; ATP was from horse muscle and NADP⁺ was prepared from yeast β -NAD⁺. All these products and DL-tryptophan were purchased from Sigma Chemical Co. D-Glucose was from Prolabo (Paris, France).

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Stationary states of glucose-6-phosphate dehydrogenase activity in a continuously stirred tank reactor

The reaction catalysed by the enzyme was analysed in a reactor operating under open conditions. The reactor was continuously fed with solutions 1, 2 and 3 (see below) at a constant flow rate (0.033 ml/min for each). Since the volume of the reactor (2 ml) was held constant, the outflow of the system was 0.1 ml/min. The reaction inside the reactor was started by adding an enzyme solution (solution 3, see below) at different pH values.

Glucose-6-phosphate dehydrogenase assay conditions in a continuously stirred tank reactor. The assay of the enzyme activity in the reactor was performed in the presence of 3.5 units of enzyme/ml (at the steady state) under conditions of zero-order kinetics at 25 °C, in 0.1 M-Hepes containing 0.1 M-MgCl₂, adjusted to pH 7.5 with NaOH solution. By mixing equal volumes of solutions 1 and 2 (see below), 1.35 mM-NADP⁺ (> 200-fold K_m) and 2 mM-glucose 6-phosphate (100-fold K_m) were present in the final volume of 2 ml.

Solution 1. NADP⁺ (2.7 mM) was dissolved in 0.1 M-Hepes containing 0.1 M-MgCl₂, pH 7.5.

Solution 2. Glucose 6-phosphate was provided to the continuously stirred tank reactor as a product of the reaction catalysed by hexokinase. The hexokinase assay mixture, 1.505 units of enzyme/ml, 4 mM-glucose and 4 mM-ATP in 0.1 M-Hepes containing 0.1 M-MgCl₂, pH 7.5, was left to stand for 0.5 h before injection into the reactor. The hexokinase activity corresponded to 100 μ mol of glucose phosphorylated/min per mg of protein under the conditions described above. The hexokinase assay did not interfere with glucose-6-phosphate dehydrogenase kinetic behaviour when analysed under coupled conditions [21].

Solution 3. Scheme 1 shows schematically the pH variations to which the glucose-6-phosphate dehydrogenase solutions were subjected. Different enzyme solutions, each containing 10.5 units/ml in 0.1 M-Hepes con-

taining 0.1 M-MgCl₂, pH 7.5, were first acidified (forward direction) with 6 M-HCl to pH 5.3, 4.0 and 2.0, and then alkalinized (reverse direction) with 6 M-NaOH to pH 4.0, 5.3, 6.0, 7.5 and 8.3.

The glucose-6-phosphate dehydrogenase solution corresponding to pH 8.3 (forward direction) was obtained by alkalinizing an enzyme solution at pH 7.5. The enzyme solution was left to stand for 1 h at the final pH value before being injected in the reactor because intrinsic protein fluorescence studies showed that the quantum yield remained stable after that time.

For example, the glucose-6-phosphate dehydrogenase at pH 4.0 (forward direction) was obtained by acidifying an enzyme solution at pH 7.5 to pH 4.0 and leaving it for 1 h before assay. The solution of glucose-6-phosphate dehydrogenase at pH 4.0 (reverse direction) was obtained by acidification of an enzyme solution at pH 7.5 to 2.0 followed by alkalinizing to pH 4.0, where it remained for 1 h before injection into the reactor.

Conformational changes of glucose-6-phosphate dehydrogenase monitored by intrinsic protein fluorescence

The protein intrinsic fluorescence was measured in two kinds of experiments: (a) global pH changes; (b) pH-history-dependent changes.

Global pH changes. As shown in Scheme 1, different glucose-6-phosphate dehydrogenase solutions (18 μ g/ml) in 0.1 M-Hepes containing 0.1 M-MgCl₂, pH 7.5, were successively acidified (forward direction) with 6 M-HCl to pH 6.0, 5.3, 4.0 and 2.0 and then alkalinized (reverse direction) with 6 M-NaOH to pH 4.0, 5.3, 6.0, 7.5 and 8.3. The glucose-6-phosphate dehydrogenase solution corresponding to pH 8.3 (forward direction) was obtained by alkalinizing an enzyme solution at pH 7.5.

The emission spectrum of each solution was recorded as a function of time, assuming the attainment of the desired pH value as time zero at 5 min, 10 min, 15 min, 30 min, 60 min and 24 h. In addition, the absorption spectrum of each solution was recorded to correct for inner-filter effects [22].

Scheme 1. Experimental scheme of successive pH variations to which the glucose-6-phosphate dehydrogenase solutions were subjected

Glucose-6-phosphate dehydrogenase solutions at pH 7.5* (initial pH) were first acidified (forward direction, light line), then alkalinized (reverse direction, heavy line), and left for 1 h at the final pH in each case. The glucose-6-phosphate dehydrogenase solution corresponding to pH 8.3 (forward direction) was obtained by alkalinizing an enzyme solution at pH 7.5. The kinetics or fluorescence of the enzyme solutions thus obtained were analysed as described in the Experimental section.

pH-history-dependent changes. A concentrated glucose-6-phosphate dehydrogenase solution (stock solution, 180 $\mu\text{g/ml}$) in 0.1 M-Hepes containing 0.1 M- MgCl_2 , pH 7.5, was subjected to successive steps of acidification (forward direction) to pH 6.0, 5.3, 4.0 and 2.0 followed by alkalization (reverse direction) to pH 4.0, 5.3, 6.0, 7.5 and 8.3 (Scheme 1). The enzyme solution corresponding to pH 8.3 (forward direction) was obtained by alkalizing a portion of the stock solution (pH 7.5). At each step 0.3 ml of the stock solution was diluted with 2.7 ml of 0.1 M-Hepes containing 0.1 M- MgCl_2 , pH 7.5.

The fluorescence spectra of the different solutions were monitored as a function of time, considering the time of dilution as time zero, at 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 60 min and 24 h. The absorption spectrum of each solution was also measured to allow the correction for inner-filter effects [22]. These two kinds of experiments (*a* and *b*) attempted to simulate the conformational changes undergone by the enzyme before and after injection into the open reactor respectively.

Fluorescence measurements. Intrinsic glucose-6-phosphate dehydrogenase fluorescence was measured with a Perkin-Elmer luminescence spectrometer LS-5. The protein was excited at 285 nm (excitation bandwidth 2.5 nm). Relative fluorescence intensity in each experimental condition (see above) was corrected for inner-filter effects by the method proposed by Brand & Wiholtz [22].

The emission spectra were recorded between 300 and 450 nm (emission bandwidth 2.5 nm). The emission monochromator was rotated at a rate of 10 nm/min. The correct emission spectra were integrated by weighing the area under the curve. The quantum yield of glucose-6-phosphate dehydrogenase at each experimental condition was calculated from the ratio of the integrated emission spectrum of the enzyme to that of a tryptophan solution (5.1 $\mu\text{g/ml}$) in 0.1 M-Hepes containing 0.1 M- MgCl_2 , pH 7.5.

RESULTS AND DISCUSSION

Bistability in the reaction catalysed by glucose-6-phosphate dehydrogenase in an open reactor

The activity of glucose-6-phosphate dehydrogenase was measured as stationary values of NADPH produced in an open reactor. Bistable behaviour was obtained for the activity of the enzyme in solutions treated at different pH values (Fig. 1). The reaction pH of 7.5 and measurement conditions inside the reactor were kept constant so that only the pH of the enzyme solution that was fed to the reactor (solution 3) varied from measurement to measurement. The glucose-6-phosphate dehydrogenase activity was maximal when the enzyme was at its optimal pH of 7.5 before being injected into the reactor. As the enzyme solution was acidified (forward direction) the steady-state value of NADPH decreased progressively for enzyme solutions down to pH 2.0. The following alkalization of the enzyme solutions did not produce a recovery of the corresponding steady-state values, observed during the acidification steps, until the solution reached pH 8.3. Thus the activity of glucose-6-phosphate dehydrogenase, reflected by the steady state concentration of NADPH, depended not only on the pH of the enzyme solution injected into the reactor but also on its history.

Fig. 1. Stationary values of NADPH obtained as result of glucose-6-phosphate dehydrogenase activity (pH-history-dependent) in the continuously stirred tank reactor

The reaction catalysed by glucose-6-phosphate dehydrogenase was analysed in a reactor operated under open conditions (see the Experimental section). The absorbance at 340 nm of the reactor outlet flow was measured and registered continuously with a Beckman spectrophotometer. Steady-state NADPH concentration was considered to be attained when the variation in A_{340} was less than 0.02/h. The time necessary for achieving the stationary values of NADPH was 2 ± 0.5 h, except for pH 8.3, either forward or reverse, which required 1 ± 0.15 h. For calculating the concentration of NADPH at the steady state a molar absorption coefficient of $6200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used. The pH of the enzyme solution that was fed to the reactor was varied in the forward (□) or the reverse (■) direction up to the indicated pH values before injection into the reactor (see the Experimental section). Broken lines show the hypothetical pathway followed by glucose-6-phosphate dehydrogenase activity between both steady-state branches.

In addition, the initial rate of the reaction catalysed by glucose-6-phosphate dehydrogenase showed bistable behaviour when analysed with respect to pH. Fig. 2 shows that close to the isoelectric point, pH 5.4 [23], a higher initial rate was observed than at pH 7.5, although the stationary value of NADPH produced was lower at pH 5.3 than at pH 7.5. At pH 5.3 (forward direction) the temporal evolution of NADPH absorbance showed two distinct slopes before reaching the steady state; in this case it was the first slope that was used for the calculation of the initial rate (Fig. 2). It is remarkable that, although the stationary values of NADPH achieved by the system are quantitatively large, principally in the forward direction, the initial rates for attaining them are rather low, independent of the direction of pH change (Fig. 2).

Two steady states of activity (bistability) for each pH value in the open continuously stirred tank reactor were observed upon successive pH variations of the glucose-6-phosphate dehydrogenase solution. The bistable behaviour was reflected either by the total NADPH produced at steady state or by the initial rate of the enzymic reaction catalysed by glucose-6-phosphate dehydrogenase.

Conformational changes of glucose-6-phosphate dehydrogenase under the influence of pH

Although the enzyme under study did not show an autocatalytic effect and was homogeneously distributed by continuous stirring, bistable behaviour was shown to

Fig. 2. Initial-rate behaviour of the reaction catalysed by glucose-6-phosphate dehydrogenase in an open continuously stirred tank reactor (pH-history-dependent)

The initial rate of the reaction in the open reactor was determined from the slope of the register obtained at each pH value (see Fig. 1 legend). In all cases a variable lag time (4–15 min) was observed. The successive pH changes in the forward (□) and the reverse (■) directions of the enzyme solution that was fed to the reactor and the operating conditions were as described for Fig. 1.

Fig. 3. Glucose-6-phosphate dehydrogenase quantum-yield behaviour through successive pH changes

Quantum-yield values of enzyme solutions (18 μ g/ml) were measured at each pH, after successive changes in the forward (□) and the reverse (■) directions. Quantum-yield values were obtained by integration of the corrected fluorescence emission spectra with respect to the corresponding emission spectrum of a tryptophan solution (see the Experimental section).

exist. Thus a structural hysteresis in the molecule would be the only way to store information on the pH history of the enzyme.

Global pH changes

The pH-dependent conformational changes in glucose-6-phosphate dehydrogenase were studied by intrinsic protein fluorescence. Fig. 3 shows the behaviour of the quantum yield when the pH of the enzyme solution was successively varied in the forward and reverse directions. A decrease of 31 % in protein fluorescence was observed when the pH was changed from 4.0 down to 2.0. This would indicate that an important conformational change occurred in that pH region. This latter conformational

Fig. 4. Fluorescence intensity of glucose-6-phosphate dehydrogenase upon successive pH changes

The pH of an enzyme solution was successively adjusted in the forward (□) and the reverse (■) directions up to the indicated value. For each solution the corrected fluorescence intensity at the wavelength of maximal emission was measured (see Table 1 and the Experimental section). Inset shows the fluorescence emission spectra of glucose-6-phosphate dehydrogenase solutions at pH 7.5 (1) and 2.0 (2) (excitation at 285 nm). The fluorescence was monitored between 300 and 450 nm.

change could not be reversed by an increase in the pH of the solution, since the quantum-yield values of the reverse direction remained 31 %, on average, below those of solutions with the same pH but produced in the forward direction. A decrease in the quantum yield of the enzyme solutions at the isoelectric point was also noticed for both directions.

On the other hand, the corrected fluorescence intensity at the maximal wavelength emission increased rather abruptly at pH 4.0 in the forward direction, this increase being due to an increase in the absorbance of the protein solution (Fig. 4). An increase in the corrected fluorescence intensity in the reverse direction was also observed, but at pH 5.3. This change reflects precipitation of the enzyme around its isoelectric point [23]. Thermodynamically, the association–dissociation process occurring around the glucose-6-phosphate dehydrogenase isoelectric point could be interpreted as a phase transition [17]. As for other macromolecular phase transitions, the aggregation of the enzyme exhibits hysteresis, since a shift was observed when the critical pH was approached from the upper or the lower direction, suggesting the existence of metastable states.

These results indicate that the conformation of the enzyme at the time of entrance into the reactor was different, and depended on the direction of the pH changes (forward or reverse). When the enzyme entered the reactor it apparently retained in its conformation the previous pH record (Fig. 5). We remark ‘apparently’ since the intrinsic protein fluorescence changes have been simulated with the isolated enzyme and it is known that conformational changes of glucose-6-phosphate dehydrogenase occur when it interacts with its substrates [24].

Table 1 shows the shift in the maximal emission wavelength when the pH of the enzyme solution was successively changed (global pH changes). A shift of

Fig. 5. pH-history-dependent changes of glucose-6-phosphate dehydrogenase quantum yield

The pH of the glucose-6-phosphate dehydrogenase stock solution was successively adjusted in the forward (●) and the reverse (○) directions up to the indicated value. A sample of the stock solution at each step was diluted with 9 vol. of 0.1 M-Hepes/NaOH containing 0.1 M-MgCl₂, pH 7.5. The quantum yield was determined 60 min after dilution by comparing the integrated emission spectrum corrected for inner-filter effects with the corresponding corrected spectrum of tryptophan (see the Experimental section).

Table 1. Maximal emission wavelength through pH variations

Glucose-6-phosphate dehydrogenase solutions (18 µg/ml) at the indicated final pH after successive pH changes in the forward and reverse directions (global pH changes) were examined. The fluorescence emitted between 300 and 450 nm (excitation at 285 nm) was recorded and the wavelength of maximal intensity was determined for each solution.

pH (forward)	Maximum emission wavelength (nm)	pH (reverse)	Maximum emission wavelength (nm)
8.3	349	4	343
7.5	349	5.3	345
6.1	346	6.1	347
5.3	347	7.5	345
4	343	8.3	348
2	343		

6 nm was observed for solutions in the range of acidic pH (2.0 and 4.0) with respect to neutral pH. The same shift was observed when portions of the glucose-6-phosphate dehydrogenase stock solution at pH 4.0 and 2.0 were diluted in 0.1 M-Hepes containing 0.1 M-MgCl₂, pH 7.5 (results not shown).

pH-history-dependent changes

A glucose-6-phosphate dehydrogenase solution (180 µg/ml) was cycled to different pH values and samples were extracted and placed in a solution of 0.1 M-Hepes containing 0.1 M-MgCl₂, pH 7.5, in which the enzyme showed its maximum activity. A time-dependent change in quantum yield was observed that reached stationary values after 60 min.

When the stationary value of quantum yield was plotted versus pH, a hysteresis loop was obtained (Fig. 5). Unlike the behaviour of the maximal emission wavelength, the quantum yield showed a pronounced difference when the enzyme was cycled in the reverse direction (Fig. 5). At pH 8.3 (forward direction) a large decrease in quantum yield was noticed. The quantum yield of glucose-6-phosphate dehydrogenase was significantly different for reverse cycling (alkalinization) with respect to forward cycling (Figs. 3 and 5) for both global pH changes and pH-history-dependent changes.

Since the intrinsic fluorescence emission spectrum of glucose-6-phosphate dehydrogenase was apparently due to tryptophan residues ([25]; see Fig. 4 inset), the different quantum yield values would indicate changes in the conformational state of the protein [26,27]. The relative fluorescence quenching as judged by changes in quantum yield would correspond to different degrees of exposure of tryptophan residues. An increase in quantum yield would indicate that tryptophan residues are less 'exposed' to the solvent [28]. The energetic status of tryptophan residues at low excitation wavelengths (260–280 nm) is more heterogeneous than at higher ones (results not shown). The quantum yield of the protein excited at 300 nm was low, suggesting that only a small and energetically homogeneous tryptophan population could emit the absorbed light as radiation energy.

The hypothesis that the enzyme has undergone acid denaturation at pH 2.0, which would result in an irreversible loss of activity, can be discarded from the fact that the same enzyme solution that was first adjusted to pH 2.0 (eliciting a decrease in the activity) was later alkalinized to pH 8.3, whereupon full enzymic activity was recovered. Therefore these results are better interpreted as a consequence of a reversible ionization of the protein groups. Thus fluorescence measurements clearly indicate conformational changes associated with pH variations (Figs. 3–5). Stable or at least long-lived metastable conformational states of the enzyme give rise to bistability. Unlike other examples of conformational transitions undergone by biopolymers, the activity states cannot be attributed to time-dependent slow transitions but are rather to be attributed to stable conformational changes or at least long-lived metastable states [16].

Correlation between conformational and activity steady-state values of glucose-6-phosphate dehydrogenase

In Fig. 6 the enzyme activity is correlated with conformational changes observed through protein intrinsic fluorescence after pH-history-dependent changes. It appears that between pH 8.3 and 5.3 there is no correlation between the enzyme activity in the continuously stirred tank reactor and quantum yield. However, after the pH corresponding to the isoelectric point of glucose-6-phosphate dehydrogenase (forward direction), the enzyme activity and its conformation showed a strong interdependence. Another point of inflexion occurs at pH 4.0 (reverse direction), after which activity and conformation are also tightly coupled. It is remarkable that after pH 5.3 (forward direction) quantum yield always decreases. A complex ensemble of states of a protein includes a series of potential-energy minima, each one separated by an energetic barrier, only some of which are enzymically active [29]. That this could be the case for the present study is shown in Figs. 1, 5 and 6, since for different quantum yields, depending on the pH history of

Fig. 6. Kinetic-structural relationships of glucose-6-phosphate dehydrogenase (pH-history-dependent changes)

NADPH concentrations at steady state (see Fig. 1) are plotted against quantum yield of the enzyme (see Fig. 5) at each pH value, indicated in parentheses, in the forward (●) and the reverse (○) directions.

the enzyme, different activities and correlations between activity and conformation were corroborated. The results described above reflect the fact that different conformations may have the same activity; conversely, the activity level may suffer abrupt changes when relatively small conformational changes occur. Therefore the fluorescence of tryptophan residues in the protein appears to arise from different domains, associated or not with the enzyme active site.

We suggest that both conformational changes and alterations in intermolecular associations are the non-linear mechanism that gives rise to the dynamic bistable behaviour in the experiment in the continuously stirred tank reactor (Fig. 6). That a conformational change in an enzyme could have dynamic consequences has been demonstrated in an open futile cycle composed of two enzymes [30]. The pH-induced process of glucose-6-phosphate dehydrogenase folding-unfolding (intramolecular) and association-dissociation near the isoelectric point (intermolecular) involving covalent (e.g. disulphide) bonds and non-covalent (e.g. electrostatic, hydrogen and hydrophobic) bonds would be the information-transducing mechanism of the enzyme's record of previous pH [31,32]. When this structural stored information is transduced, a bistable dynamic behaviour appears in an open reactor (Figs. 1 and 6).

One of the unanswered questions in metabolism is whether, if enzymes are organized in complexes, the formation and dissolution of such complexes may be involved in metabolic regulation [33,34]. The present study raises the possibility of this kind of regulation, through multiple steady states resulting from changes in intermolecular association-dissociation equilibria. A macromolecular bistable network would give rise to alternative paths of metabolic regulation by changing the flux between two stationary states along the pathway concerning the enzyme(s) implicated.

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