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Polarised pulse fluorimetry study on the conformational properties of wheat germ hexokinase LI

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Abstract. The conformational properties of wheat germ hexokinase LI, a monomeric enzyme showing non-Michaelian kinetics, have been studied by polarised pulse fluorimetry using synchrotron radiation as an excitation light source.

The fluorescence decays and the fluorescence anisotropy decays of tryptophyl residues were measured with excitation at 300 nm. At pH 8.5, we found that the "mnemonical" temperature-dependent transition did not induce any detectable structural change in the protein. This rules out modifications of the aggregation state of hexokinase during the transition as well as important conformational changes in the tertiary structure. At pH 6.1, a temperature-dependent transition of the enzyme-glucose binary complex is observed: rapid, large amplitude, internal motions appear in the structure when the temperature is raised from −1 °C to 30 °C. Full standard activity is retained during this dynamic change.

In the experiments described here we obtained an active fluorescent derivative by reacting hexokinase with N-(iodoacetylaminoethyl)-5-naphtylamine-1-sulfonic acid (1,5-IAEDANS), in the presence of glucose. Polarised fluorescence decay measurements indicate that the label is exposed to the solvent and very mobile, which makes it ineffective as a probe for the conformational properties of hexokinase.

Key words: Hexokinase (wheat germ), conformational change, hysteresis, pulse fluorimetry, synchrotron radiation

Introduction

Wheat germ hexokinase (E.C. 2.7.1.1) is a monomer of M.W. 50,000 with a single active site for each of its substrates (Meunier et al. 1974; Higgins and Easterby 1974). Kinetic studies performed on isoenzyme LI by Meunier et al. (1974) have shown negative cooperativity at pH 8.5 towards the first substrate, glucose, and Michaelian behaviour towards the second substrate, MgATP. No self-association of hexokinase has been observed by gel filtration and sedimentation equilibrium experiments (Meunier et al. 1971, 1974; Higgins and Easterby 1974). Therefore, this behaviour cannot be simply explained in terms of allosteric interactions, and an hysteretic model has been proposed (Ricard et al. 1974), which was called the "mnemonical" model. The mnemonical mechanism is in good agreement with kinetic data obtained from three monomeric enzymes: wheat germ hexokinase (Meunier et al. 1974), rat liver glucokinase (Storer and Cornish-Bowden 1977) and octopine dehydrogenase from *Pecten maximus L.* (Monneuse-Doublet et al. 1978).

The mnemonical enzyme concept (Fig. 1) is a particular case of the more general hysteretic concept developed by Ainslie et al. (1972) to account

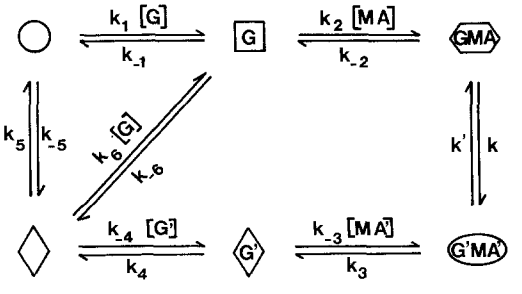

Fig. 1. The mnemonical model for wheat germ hexokinase LI (from Meunier et al. 1974). G, MA, MA′ and G′ represent respectively, glucose, MgATP, MgADP and glucose-6-phosphate

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Abbreviations: 1,5-IAEDANS N-(iodoacetylaminoethyl)-5-napthylamine-1-sulfonic acid; DTNB 5,5′-dithiobis(2-nitrobenzoic acid)

for regulatory behaviour of monomeric enzymes, as well as the particularly complex kinetics observed with some oligomeric enzymes. The mnemonical model assumes that the free enzyme exists in (at least) two catalytically different and interconvertible conformations. Only one of these conformations being restorted after one catalytic cycle, the conformational equilibrium is thus shifted during catalysis, leading to steady-state cooperativity and transients during fast kinetic studies. Furthermore, the conformational equilibrium may be shifted by temperature: a slow response in the steady-state fluorescence intensity of hexokinase has been monitored at pH 8.5 after *T*-jumps from 2 °C to 35 °C (Buc et al. 1977). The corresponding time constant of the relaxation (the "mnemonical" transition) shows the same dependency upon ligand concentration as the relaxation detected during fast kinetic studies.

Polarised pulse fluorimetry allows simultaneous measurements of fluorescence lifetimes and Brownian rotational correlation times of macromolecules in solution. Thus, the method provides both static information about local dielectric constants in a macromolecular structure, and dynamic information about rotational motions in the nanosecond time scale. Previous studies, using this technique, have shown that tryptophyl residues of proteins may exhibit a wide range of dynamic behaviour (Munro et al. 1979). Our purpose was to check the possible effects of hexokinase conformational changes on these structural and dynamic parameters. Polarised fluorescence decays were measured using the synchrotron radiation produced by an electron storage ring, which gives accurate measurements in short times for relatively low protein concentrations.

We report here the effect of temperature on the polarised fluorescence decays of tryptophyl residues of hexokinase LI. At alkaline pH's, the "mnemonical" transition does not result in any detectable change in the environment and mobility of tryptophyl residues, whereas at pH 6.1, a dynamic transition of the enzyme-glucose binary complex occurs upon raising the temperature.

A fluorescent reagent, AEDANS, was covalently attached without loss of activity to the most accessible sulfhydryl group of hexokinase. However, because of the intrinsic mobility of the label, it was unable to probe the molecular dynamics of the protein. This highlights a general constraint on labelling procedures for pulse fluorimetry studies.

Materials and methods

Materials

Wheat germ was purchased from local sources. Glucose-6-phosphate dehydrogenase, NADP, ATP were purchased from Boehringer; glucose, N-ethylmaleimide, *p*-chloromercuribenzoate from Merck; bovine serum albumin, DTNB and 1,5-IAEDANS from Sigma. Commercial reagents were used without further purification.

Methods

Hexokinase purification. We purified hexokinase following Meunier et al. (1971) and Meunier (1974). A crude extract was first obtained by stirring and centrifugation of wheat germ in a 50 m*M* phosphate buffer pH 7.5, containing 40 m*M* glucose, 5 m*M* cysteine, at 2 °C. This extract was fractionated between 50% and 70% ammonium sulfate. The last precipitate was dissolved in a 50 m*M* phosphate buffer pH 7, 40 m*M* glucose and then dialyzed for half an hour in 100 m*M* acetate buffer pH 5, 40 m*M* glucose. The dialysis was continued for several hours in a 5 m*M* succinate buffer pH 5.8, 40 m*M* glucose. The extract was then applied to a DEAE-cellulose column equilibrated with the same buffer, and eluted at 6 °C by a linear KCl gradient. Two major peaks of hexokinase activity are eluted, numbered FI and FII according to the order of elution. Peak FI was pooled and concentrated, and then sieved on a Sephadex G-100 column, equilibrated with a 50 m*M* phosphate buffer, pH 7, 40 m*M* glucose, 100 m*M* KCl, at 6 °C. The light form, LI, is thus separated from the heavy form, HI.

This method, designed for biochemical studies, gave very poor yields of enzyme (1 to 2 mg for the treatment of 1 kg of wheat germ), and led to a very unstable product, resulting from unseparated traces of proteases. Our preparations had to fulfill high (i.e. spectroscopic) concentration requirements, and thus, had to be carried out on a large scale, to the prejudice of chromatographic resolution. With such preparations, although they yielded good specific activities as compared with previously published values (Table 1), it was difficult to obtain the enzyme in pure form. Contaminating components were distributed over eight different molecular species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were estimated to be less than 25% of the total protein content. Thus, the results presented in this paper will be valid only if none of these contaminants has a high quantum yield associated with an important temperature dependency. Proteolytic activity in our extracts was studied, and found to be sensitive to thiol reagents. However, hexokinase activity is also, as will be seen later, sensitive to thiol reagents.

Analytical biochemical methods: Hexokinase activity was assayed by coupling the enzyme to glucose-6-

Table 1. Specific activity and yields of hexokinase purification

	Values obtained in our preparations		Previously published values[a]	
	Specif. activ. [U/mg]	Yield [%][c]	Specif. activ. [U/mg]	Yield [%][c]
Crude extract	0.006[b]	100%	0.005[b]	100%
Soluble fraction at 50% Amm. Sulf.	0.008[b]	44%		
Precipitate at 70% Amm. Sulf.	0.016	42%	0.012	46%
Dialysate	0.065	34%	0.045	45%
DEAE chromatography [FI pool]	0.41	2%	0.17	2.5%
Molecular sieving [LI peak]	1.3	0.4%	0.70	0.4%

[a] Meunier JC (1974) Doctoral thesis
[b] Corrected values for 6-phospho-gluconate dehydrogenase activity following Meunier
[c] Percent of the total hexokinase activity in the crude extract

phosphate dehydrogenase. The reduction of NADP was followed by absorption at 340 nm, 30 °C in a 125 m*M* triethanolamine buffer pH 8, containing 10 m*M* glucose, 0.3 m*M* ATP, 0.8 m*M* MgCl, 0.08 m*M* NADP and 0.2 units/ml of glucose-6-phosphate dehydrogenase. Protein content was assayed following Bradford (1976), using bovine serum albumin as a standard. Electrophoresis under denaturating conditions was realised on 10% sodium dodecyl sulfate-polyacrylamide gel as described by Weber and Osborn (1969). Thiol reactivity was assayed following Ellmann's method (1959).

Reaction of hexokinase with 1,5-IAEDANS: The incubation solution was a 50 m*M* phosphate buffer pH 7, containing 100 m*M* KCl, 40 m*M* glucose, 0.02 m*M* hexokinase and 0.08 m*M* 1,5-IAEDANS. It was stored in the dark for 4 h at 4 °C, and then applied to a Sephadex G-25 column, equilibrated with a 100 m*M* triethanolamine buffer pH 8.5, 40 m*M* glucose. The final concentration of the dye was estimated using a molar absorption coefficient of $6{,}100\ l \cdot M^{-1} \cdot cm^{-1}$ at 337 nm for AEDANS, as reported by Hudson and Weber (1973).

Absorption and fluorescence spectra: Enzymatic assays, optical densities and absorption spectra were measured with a double beam Perkin-Elmer 555 spectrophotometer. Fluorescence spectra were obtained with a Jobin-Yvon JY3N spectrofluorimeter.

Pulse fluorimetry measurements: The fluorescence decays were measured using the single photo-electron counting method (Yguerabide 1972; Wahl 1975). The excitation pulsed light source was the synchrotron radiation emitted by the ACO storage ring at LURE, Orsay, France. This light beam is emitted in near gaussian pulses (1.8 ns measured fwhm) at a frequency of 13.6 MHz in a single bunch mode, and spreads from soft X-rays to infrared. The detection apparatus includes an SLM 8000 spectrofluorimeter, a Philips XP2020Q photomultiplier and conventional electronic modules for photon counting, connected to a micro-computerised multichannel analyser (Nuclear Data ND660). The wavelength of excitation is selected by a double grating monochromator and fluorescence from the sample is detected, at a 90° angle to the incident beam direction, through MTO interference filters. Other details of the experimental procedure were as previously described (Jameson and Alpert 1979; Brochon 1980).

Polarised fluorescence decays of the tryptophyl residues of hexokinase were measured with excitation at 300 nm ($\Delta\lambda$ 4 nm). This wavelength corresponds to a high fundamental anisotropy (0.30) of tryptophyl fluorescence (Valeur and Weber 1977), and thus gives more accurate anisotropy measurements. The free enzyme (0.4 μ*M* hexokinase) was studied in 100 m*M* triethanolamine buffer, pH 8.5, at −1°, 10°, 20°, 30 °C. The fluorescence was detected through an interference filter centered at 358 nm ($\Delta\lambda$ 6 nm). The enzyme-glucose binary complex (0.2 μ*M* hexokinase) was studied in 5 m*M* succinate buffer, pH 6.1, 40 m*M* glucose, at −1 °C and 30 °C. As the affinity constant of hexokinase for glucose is $3.5 \times 10^3\ M^{-1}$ at 30 °C (Meunier et al. 1974), the binary complex represents more than 99% of the total enzyme content. The fluorescence was detected through a wide band filter centered at 359 nm.

The free enzyme is studied at pH 8.5 and the enzyme-glucose binary complex at pH 6.1, because these experimental conditions are close to those in which conformational changes of the enzyme have been reported (Meunier et al. 1974; Buc et al. 1977; Buc 1977). Consequently, the experiments on the free enzyme and on the binary complex should be compared with caution. The reversibility of the transition between −1 °C and 30 °C was checked at pH 8.5 without glucose, because hexokinase has a particular tendency to become inactivated in the absence of its substrate (Meunier 1974; Higgins and Easterby 1974).

Polarised fluorescence decays of the AEDANS-enzyme-glucose binary complex were measured at −1 °C and 30 °C, in 100 m*M* triethanolamine buffer pH 8.5, 40 m*M* glucose, 5 μ*M* hexokinase. The wavelength of excitation was 350 nm ($\Delta\lambda$ 16 nm). The fundamental anisotropy of AEDANS at this excitation wavelength is 0.38, and corresponds to a

single absorption band (Hudson and Weber 1973). The fluorescence was detected through an interference filter centered at 520 nm ($\Delta\lambda$ 4 nm).

Theory

Analysis of experimental fluorescence decays

The observed transient fluorescence, $i_{exp}(t)$, is related to the fluorescence emitting law $I(t)$ by a convolution product:

$$i_{exp}(t) = \int_0^t g(T)\, I(t-T)\, dT = g(t) * I(t)\,, \qquad (1)$$

where $g(t)$ is the instrumental response function. Synchrotron radiation is continuously tunable, and the time structure of the light pulse is independent of the wavelength (Bénard and Rousseau 1974). Therefore, the variation of the temporal response $g(t,\lambda)$ of the apparatus with wavelength is only a function of the photomultiplier colour response (Wahl et al. 1974). The instrumental response function can thus be measured at each emission wavelength with a scattering solution (LUDOX) in the sample compartment. In the case of AEDANS fluorescence, three consecutive pulses were taken into account in the convolution procedure to correct for the remaining fluorescence resulting from preceding excitation pulses.

The fluorescence emitting law was assumed to be a sum of N exponential functions:

$$I(t) = \sum_{i=1}^{N} A_i \exp^{-t/\tau_i} \qquad (2)$$

τ_i and $A_i = K c_i$ ($K = \sum A_i$ so that $\sum c_i = 1$) values were determined by a non-linear least-squares method (Grinvald and Steinberg 1974). A mean lifetime is defined by the relation:

$$\bar{\tau} = \sum c_i \tau_i^2 / \sum c_i \tau_i\,. \qquad (3)$$

As $i_{exp}(t)$ is always obtained by measuring the polarised components $i_\parallel$ and $i_\perp$ ($i_{exp}(t) = i_\parallel(t) + 2 i_\perp(t)$, see below), the correctness of the calculated parameters was checked by the following deviation function (Wahl 1979):

$$\mathrm{Dev}(\tau) = (i_{exp}(t) - i_{cal}(t))/\sqrt{i_\parallel(t) + 4 i_\perp(t)} \qquad (4)$$

and by the weighted residual:

$$\chi^2 = \frac{1}{n-p} \sum_{t=1}^{n} \mathrm{Dev}(t)^2 \qquad (5)$$

n being the total number of channels on the time scale, and p the number of fitted parameters. Given the calculated parameters, τ_i and c_i, the relative participation P_i of each component τ_i to the overall intensity $F(\lambda)$, measured with a continuous excitation, can be determined:

$$P_i(\lambda) = c_i(\lambda)\, \tau_i / \sum c_i(\lambda)\, \tau_i\,. \qquad (6)$$

Analysis of experimental fluorescence anisotropy decays

The sample is excited by a vertically polarised pulse of light. The two principal components of the transient fluorescence, $i_\parallel(t)$ and $i_\perp(t)$, and measured separately and alternatively. The fluorescence anisotropy decay is defined by:

$$R(t) = (I_\parallel(t) - I_\perp(t))/(I_\parallel(t) + 2 I_\perp(t)) = D(t)/S(t)\,. \qquad (7)$$

The curve:

$$s(t) = i_\parallel(t) + 2 i_\perp(t) = g(t) * S(t)\,. \qquad (8)$$

corresponding to the total fluorescence decay, is first analysed as previously described. The experimental function:

$$d(t) = i_\parallel(t) - i_\perp(t) = g(t) * D(t) \qquad (9)$$

is then analysed by a non linear least-squares method (Wahl 1979) knowing that:

$$D(t) = S(t)\, R(t) = \sum_{i=1}^{N} \sum_{j=1}^{M} A_i r_j \exp^{-t(1/\tau_i + 1/\theta_j)}\,, \qquad (10)$$

where A_i, τ_i are the calculated parameters of $S(t)$, and r_j, θ_j are the parameters of $R(t)$, which is assumed to be a sum of M exponentials:

$$R(t) = \sum_{j=1}^{M} r_j \exp^{-t/\theta_j} \quad \left(\text{with} \sum_{j=1}^{M} r_j = r_0\right) \qquad (11)$$

r_0 is the initial anisotropy of fluorescence. For the case of a rigid sphere rotating a homogeneous medium, the anisotropy decay law is a simple exponential, with time constant, θ, equal to the *Brownian rotational correlation time* of the molecule, defined by the Stokes-Einstein relation:

$$\theta = \eta V / kT \qquad (12)$$

η being the viscosity of the solvent, V the volume of the sphere, T the temperature, and k Boltzmann's constant.

Results

1. Tryptophyl fluorescence of hexokinase

a) Fluorescence spectra of hexokinase were recorded at 20 °C, with excitation at 296 nm ($\Delta\lambda$ 10 nm) (data not shown). The maximum of fluorescence of the non-corrected spectra was centre short wavelength: 335 nm (full width at half maximum 55 nm).

b) Fluorescence decays: Hexokinase contains seven tryptophyl residues (Buc et al. 1977). It is not surprising, therefore, that the fluorescence decays do not correspond to single exponentials. In all cases, a sum of at least two exponential functions is necessary to give a good representation of experimental data (Fig. 2).

On the free enzyme at pH 8.5, raising the temperature reduces the fluorescence lifetimes, which is an expected thermal effect (Table 2). The proportion of the different components remains constant. Thus, heterogeneity of the fluorescence is not affected by temperature. The fluorescence decay of hexokinase at −1 °C, after 5 h at 30 °C is similar to that of the protein before this treatment.

On the enzyme-glucose binary complex at pH 6.1, the thermal effect on fluorescence kinetics is also observed. But in this case, the composition of the fluorescence is modified by temperature, the contribution of the fast component going from 52% at −1 °C to 70% at 30 °C and the corresponding relative contribution to the overall intensity from 23% to 38% (Table 2).

c) Fluorescence anisotropy decays of hexokinase in most cases (except for pH 6.1, 30 °C) give good fits with single exponential functions (Fig. 3). When possible, analysis with two exponentials yields a

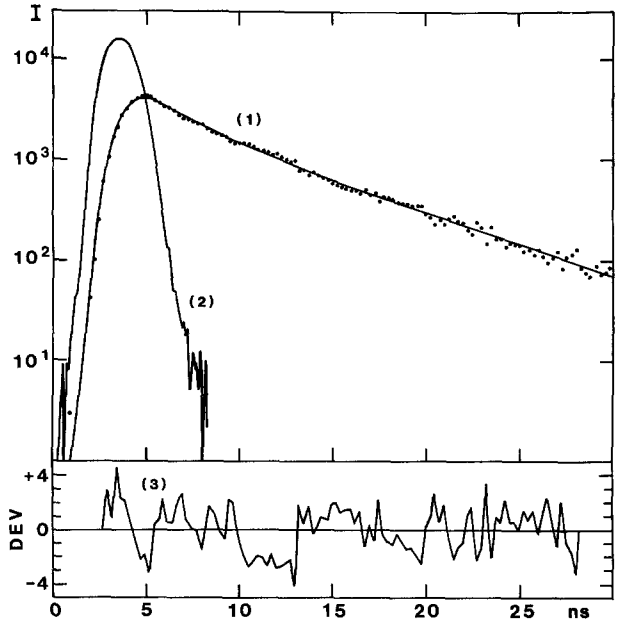

Fig. 2. Fluorescence decay of tryptophyl residues of the hexokinase-glucose binary complex, pH 6.1, −1 °C. *(1)* — calculated curve, ···· experimental data (each point is the mean of three channels on the time scale). *(2)* Instrumental response function $g(t)$. *(3)* Deviation function with a sum of two exponentials. Parameters of the calculated curve and experimental conditions are given in Table 2

Table 2. Analysis of fluorescence decays of tryptophyl residues of hexokinase. Excitation wavelength 300 nm ($\Delta\lambda$ 4 nm). Free enzyme: 100 m*M* triethanolamine buffer pH 8.5, 0.02 mg/ml hexokinase, emission through an interference filter centred at 358 nm ($\Delta\lambda$ 6 nm). Enzyme glucose binary complex: 5 m*M* succinate buffer pH 6.1, 40 m*M* glucose, 0.01 mg/ml hexokinase, emission through a wide band filter centred at 359 nm. Uncertainties in the preexponential factors are less than 5%

Free enzyme

pH	T [°C]	c_1	τ_1 [ns]	P_1	c_2	τ_2 [ns]	P_2	χ^2
8.5	−1	0.60	1.3 ± 0.2	22%	0.40	6.9 ± 0.1	78%	5.0
	10	0.60	1.5 ± 0.1	25%	0.40	6.8 ± 0.1	75%	2.6
	20	0.60	1.4 ± 0.1	25%	0.40	6.4 ± 0.1	75%	2.5
	30	0.65	1.1 ± 0.1	26%	0.35	5.9 ± 0.1	74%	1.9
	−1[a]	0.56	1.4 ± 0.1	21%	0.44	6.8 ± 0.1	79%	2.2

Enzyme-glucose binary complex

pH	T [°C]	c_1	τ_1 [ns]	P_1	c_2	τ_2 [ns]	P_2	χ^2
6.1	−1	0.52	1.8 ± 0.1	23%	0.48	6.6 ± 0.1	77%	4.0
	30	0.70	1.5 ± 0.3	38%	0.30	5.7 ± 0.3	62%	4.4

[a] Reversibility from 30 °C to −1 °C. c_i, P_i, τ_i, χ^2, are defined in the experimental procedure

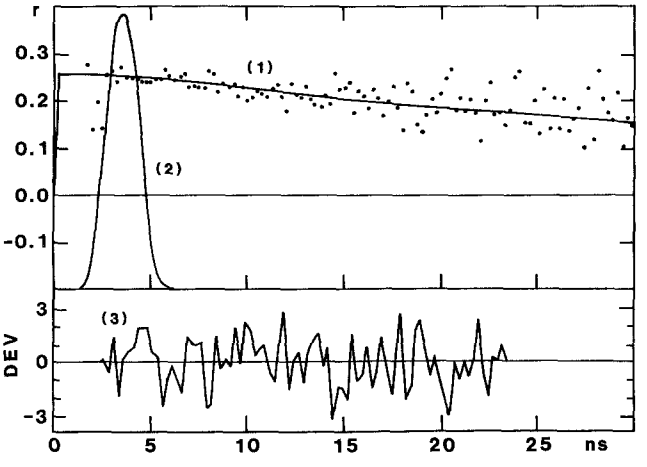

Fig. 3. Fluorescence anisotropy decay of tryptophyl residues of the hexokinase-glucose binary complex, pH 6.1, −1 °C. *(1)* — calculated curve, ···· experimental data (each point is the mean of three channels on the time scale). *(2)* Instrumental response function $g(t)$. *(3)* Deviation function with a single exponential. Parameters of the calculated curve and experimental conditions are given in Table 4

second very short time constant of less than 0.1 ns, associated with higher or equal uncertainty, and therefore seems to have no real significance.

For the free enzyme at pH 8.5, the mean correlation time is 51 ± 3 ns at −1 °C, and 23 ± 2 ns at 30 °C (Table 3). The correlation time thus decreases with increasing temperature as expected from the $\eta(T)/T$

Table 3. Analysis of fluorescence anisotropy decays of tryptophyl residues of free hexokinase. The experimental conditions are given in Table 2

pH	T [°C]	r_0	θ [ns]	χ^2
8.5	−1	0.25	51.1 ± 2.8	1.4
	10	0.25	37.6 ± 2.0	1.5
	20	0.25	25.0 ± 1.3	1.6
	30	0.23	22.9 ± 1.6	1.3
	−1 [a]	0.25	49.2 ± 3.8	1.3

[a] Reversibility from 30 °C to −1 °C. Uncertainties on the initial anisotropies are less than 1%. r_0, θ, χ^2, are defined in the experimental procedure

Table 4. Analysis of fluorescence anisotropy decays of tryptophyl residues of hexokinase-glucose binary complex. The experimental conditions are given in Table 2. Uncertainties on the initial anisotropies are less than 2% for analysis with one exponential, and 10% for the analysis with two exponentials

pH	T [°C]	r_0	θ [ns]	χ^2
6.1	−1	0.26	50.2 ± 2.6	1.0
	30	0.21	12.3 ± 0.4	2.3
(full scale)				
	30	0.15	21.6 ± 1.4	1.2
(6–25 ns)				

		r_1	θ_1 [ns]	r_2	θ_2 [ns]	χ^2
6.1	30	0.20	0.2 ± 0.1	0.17	20.0 ± 1.2	1.1

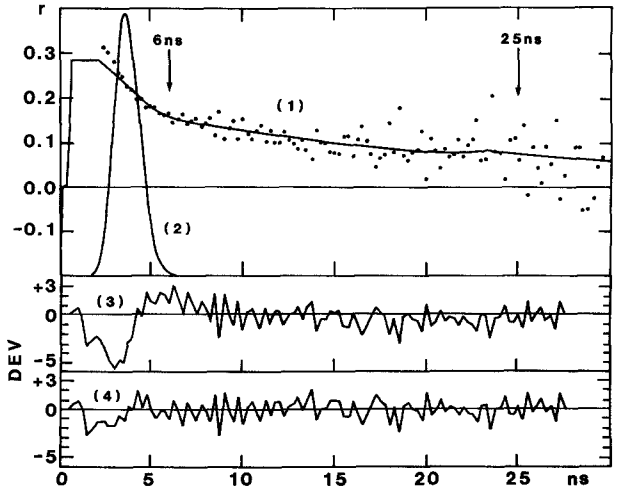

Fig. 4. Fluorescence anisotropy decay of tryptophyl residues of the hexokinase-glucose binary complex, pH 6.1, 30 °C. *(1)* — calculated curve, ···· experimental data (each point is the mean of three channels on the time scale). *(2)* Instrumental response function $g(t)$. *(3)* Deviation function with a single exponential. *(4)* Deviation function with a sum of two exponentials. Parameters of the calculated curves and experimental conditions are given in Table 4

dependency in Eq. (12). The fluorescence anisotropy decay of hexokinase at −1 °C, after 5 h of incubation at 30 °C (pH 8.5 without glucose) is similar to that of the protein before this treatment.

For the binary complex at pH 6.1, the average correlation time is 50 ± 3 ns at −1 °C, and becomes 12.3 ± 2 ns at 30 °C (Table 4). However, the adjustment to a single exponential becomes very poor for this last measurement (Fig. 4). Two exponentials are necessary to correctly represent this decay, with a long component of 20 ± 1 ns, and a short component associated with a large amplitude. In such an analysis, the initial anisotropy of the decay is higher than the fundamental anisotropy of tryptophyl residues. Therefore, some uncertainty probably affects the determination of both the short constant and its amplitude. However, the time constant obtained by analysis of the end of the decay (6 ns − 25 ns) confirms the value of the long component (Table 4 and Fig. 4).

d) Hexokinase activity remains the same, in all of the above experiments, after the pulse fluorimetry measurements (up to 5 h of incubation at 30 °C).

2. Hexokinase sulfhydryl group reactivity

1,5-IAEDANS is a fluorescent dye reacting specifically with sulfhydryl groups (Hudson and Weber 1973). We first examined the possible reaction sites of this label on hexokinase. Meunier et al. (1971) found that hexokinase contains four systeme residues. Our measurements indicate that, in the native structure, in the presence of 40 m*M* glucose, only one sulfhydryl group is accessible to DTNB. In the presence of 0.1% sodium dodecyl sulfate, two sulfhydryl groups react with DTNB. Meunier et al. (1974) found that the enzyme is not completely denaturated at this concentration of sodium dodecyl sulfate, but only "desensitized" and shows residual Michaelian activity, which was explained by an effect of sodium dodecyl sulfate on the conformation of hexokinase.

DTNB did not inactivate hexokinase during thiol assay in the presence of glucose. Under the same conditions, N-ethylmaleimide (5 m*M*) does not inactivate, but *p*-chloromercuribenzoate (2 m*M*) does. However, when glucose is removed, N-ethylmaleimide inhibits hexokinase in a few hours (and more rapidly than does the enzyme spontaneously inactivate because of the absence of glucose; Meunier et al. 1974). Therefore, some of the thiol groups of hexokinase are apparently important for the activity, and can be protected against thiol reagents by the binding of glucose. 1,5-IAEDANS was then reacted with hexokinase in the presence of saturating

concentrations of this substrate. After incubation with hexokinase, the unreacted dye was removed by gel filtration, and the protein fraction showed strong AEDANS fluorescence. The rate of labelling was estimated to be less than 0.5 moles of dye per mole of enzyme, and the derivative thus obtained remains fully active. Therefore, hexokinase has presumably been labelled on the most accessible, non-essential, sulfhydryl group.

3. Fluorescence measurements on AEDANS-hexokinase derivative

The mean lifetime of AEDANS on hexokinase is 10.8 ± 0.1 ns at -1 °C. This is close to lifetimes observed for the free dye in aqueous solution, and much lower than values usually observed when AEDANS is bound to other proteins (Ikkai et al. 1979; Wahl et al. 1978; Jullien and Garel 1981; Mérola 1981).

On the other hand, the fluorescence anisotropy decays are not single exponentials. They include a short component, associated with a high amplitude, which induces a very rapid initial depolarisation of the fluorescence emission (Fig. 5). In addition, the initial anisotropy is much lower than the expected fundamental anisotropy.

Thus, AEDANS on hexokinase seems to be located close to the solvent or close to an efficient quenching group, and its fluorescence anisotropy decays suggest large subnanosecond flexibilities. As a consequence, the overall correlation time of the complex cannot be measured precisely (Table 5),

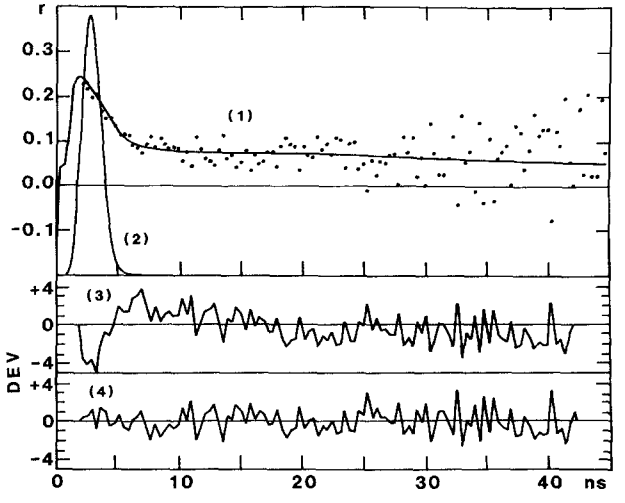

Fig. 5. Fluorescence anisotropy decay of labelled AEDANS-hexokinase-glucose binary complex, pH 8.5, -1 °C. *(1)* —— calculated curve, ···· experimental data (each point is the mean of three channels on the time scale). *(2)* Instrumental response function $g(t)$. *(3)* Deviation function with a single exponential. *(4)* Deviation function with a sum of two exponentials. Parameters of the calculated curves and experimental conditions are given in Table 5

Table 5. Analysis of fluorescence anisotropy decays of labelled AEDANS-hexokinase-glucose binary complex. Excitation wavelength 350 nm ($\Delta\lambda$ 8 nm), emission wavelength 520 nm ($\Delta\lambda$ 4 nm), 100 m*M* triethanolamine buffer pH 8.5, 40 m*M* glucose, 0.26 mg/ml hexokinase. Uncertainties on the initial anisotropies are less than 3%

pH	T [°C]	r_1	θ_1 [ns]	r_2	θ_2 [ns]	χ^2
8.5	−1	0.20	1.1 ± 0.1	0.09	77 ± 20	1.5
	30	0.15	1.1 ± 0.2	0.06	34 ± 8	1.3
	−1[a]	0.18	1.2 ± 0.1	0.09	55 ± 9	1.4

[a] Reversibility from 30 °C to −1 °C

and AEDANS thus appears to be an inefficient probe of hexokinase molecular dynamics.

Discussion

Thiol groups of wheat germ hexokinase

From our results, among the four cysteine residues of hexokinase, one sulfhydryl group seems to be particularly reactive on the native structure in the presence of substrate. This thiol group does not interfere with activity, and can react with DTNB, N-ethylmaleimide or 1,5-IAEDANS without loss of activity. This is consistent with results obtained on yeast hexokinase (Otieno et al. 1977) and on brain (type I) hexokinase (Swarup and Kenkare 1980) in which one non-essential thiol group shows high reactivity, especially when substrate or affinity reagents are bound to the active site. On the other hand, we found some thiol groups more closely involved in the activity. As Swarup and Kenkare pointed out, an active site thiol group appears to be present in most of the hexokinase investigated so far (Otieno et al. 1977; Connolly and Trayer 1979a; Swarup and Kenkare 1980), with the possible exception of the rat liver (type IV) glucokinase (Connolly and Trayer 1979b).

Tryptophyl fluorescence

As estimated by their fluorescence emission spectra, the tryptophyl residues of hexokinase are in an average hydrophobic environment. This result is also supported by the fluorescence decay measurements which indicate that the major contribution to the total fluorescence intensity is due to the slowest component. The theoretical correlation times of hexokinase (M.W. 50,000), calculated on the basis of Eq. (12) for the temperatures −1°, 10°, 20°, 30 °C, assuming an additional hydration volume of 50% (which is a minimum value observed for proteins in solution: Wahl 1980) are respectively 46, 30, 23, 18 ns (the theoretical value of θ at −1 °C is calculated by extrapolation of $\eta/T = f(T)$ for water;

the theoretical correlation time at 0 °C is 44 ns). Our experimental values are generally slightly higher. This may arise if the hydration volume is more than 50%, or if hexokinase is slightly ellipsoidal.

For the free enzyme, pH 8.5, no modification can be detected, from our experiments, during the "mnemonical" transition. Firstly, the composition of fluorescence decays remains constant when the temperature is raised. This may appear inconsistent with the previous observation of Buc et al. (1977) that the "mnemonical" transition is accompanied by a slow decrease of the total fluorescence intensity. However, measurements of fluorescence decays are made at equilibrium, and cannot discriminate between rapid thermal quenching and slow conformational effects. In addition, fluorescence decays may be insensitive to static quenching (occuring in the ground state). Conversely, a modification of the fluorescence composition versus temperature could not be strictly correlated with conformational events (Cooper 1981). However, the experimental correlation time of hexokinase varies as η/T with temperature. Thus, it is clear that the "mnemonical" transition does not result in large modifications of the structure and molecular dynamics of the enzyme. This excludes, of course, a displacement of a possible dimer-monomer equilibrium, which is a frequent alternative explanation of hysteretic kinetic behaviour (Ainslie et al. 1972; Neet and Ainslie 1980).

For the enzyme-glucose binary complex, pH 6.1, there is a differential sensitivity of tryptophyl residues to temperature, with the contribution of the fast component increasing with increasing temperature. Moreover, the experimental mean correlation time at −1 °C (50 ns) is higher than the theoretical value (46 ns) and becomes much lower (12.3 ns) than the theoretical value (18 ns) when the temperature is raised to 30 °C.

a) The hypothesis that the enzyme becomes irreversibly denaturated can be ruled out, since hexokinase was fully active after the experiment.

b) Although glucose promotes dissociation of dimeric yeast hexokinase (Colowick 1973), no self association of wheat germ hexokinase has been observed by gel filtration, between pH 5.5 and 10, up to enzyme concentrations of 17 mg/ml (Meunier 1974). The present measurements were performed with enzyme concentrations lower than 0.02 mg/ml. Therefore, the variation of the experimental correlation time of hexokinase with temperature cannot be explained by a dissociation of a dimeric form of hexokinase.

c) The long component of the fluorescence anisotropy decay (20 ns) falls between the theoretical correlation time of hexokinase at 30 °C and the corresponding experimental value obtained at pH 8.5. Therefore, this component can be ascribed to the tumbling of the whole protein. Nevertheless, a very short component associated with a high amplitude was obtained. This suggests flexibilities of lateral chains having a large degree of freedom.

Thus, our results show that hexokinase behaves, in most of the experiments, as a quasi-spherical, rigid body. However, there is evidence for a temperature-induced transition of the glucose-hexokinase binary complex, leading to important local flexibilities. The functional significance of this transition cannot, as yet, be stated. Previous kinetic studies at pH 6.9 (Buc 1977) led to the proposal of structural transitions of the enzyme-glucose binary complex during catalysis. However, it has been shown that most of the similar kinetic properties observed on yeast hexokinase are to be attributed to slow inhibition of the enzyme by aluminium ions contained in commercial samples of ATP (Neet et al. 1982). Therefore, the question remains whether such thermal transitions of enzymes (Munro et al. 1979; Blicharska et al. 1982) are not simply the first, reversible steps towards denaturation.

Conclusion

Biochemical investigations on enzymes have focussed on the "conformational change" concept. As improved physical data is collected for these phenomena, the conformational properties of enzymes seem to range from rather "subtle" rearrangements (like the hemoglobin allosteric transition: Perutz 1970) to important structural and dynamical modifications (Moody et al. 1979; Ladner et al. 1982; Jacrot et al. 1982). This is further illustrated by the present results: while the "mnemonical" transition of hexokinase at pH 8.5 has not been detected by the technique of time resolved fluorescence, the temperature induces a dynamic transition of the binary complex at pH 6.1 associated with large and rapid flexibility in the structure.

Owing to the recent advances in fast light sources, such as synchrotron radiation or picosecond lasers, polarised pulse fluorimetry is one of the different methods available today for the study of the structure and dynamics of macromolecules in solution. This could be of particular help in the physical understanding of enzyme function.

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