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Renaturation of guanidine-unfolded tryptophan synthase by multi-mixing stopped-flow dilution in D_2O

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Guanidine hydrochloride (GdnHCl) at high concentrations, e.g. 4 to 8 M, has been used extensively to promote reversible denaturation of several proteins. The refolding is induced by removal of the denaturing agent by diluting the denatured protein at least 50–100-fold in a 'renaturation buffer'. Fast kinetic studies, using a stopped-flow apparatus to achieve such dilutions, are difficult for two reasons: firstly, injecting widely different volumes of the two reagents does not afford a proper mixing. Secondly, the density differences existing between the concentrated GdnHCl solution and the renaturation buffer often causes important mixing and redistribution artefacts particularly in vertical stopped-flows. Here, it is shown that these difficulties can be overcome by using a multi-mixing stopped-flow to achieve 2 successive 7-fold dilutions and by using heavy water (D_2O) to adjust the density of the renaturation buffer. This enabled us to study the appearance of a short-lived intermediate during the folding of the β_2-subunit of *Escherichia coli* tryptophan synthase.

Stopped-flow kinetics; Deuterium oxide; Tryptophan synthase; Protein folding; Early intermediate

1. INTRODUCTION

Guanidinium chloride (GdnHCl) and urea are two denaturing agents extensively used to denature proteins [1]. Many studies on folding start from GdnHCl unfolded proteins because the denatured polypeptide chain is soluble and devoid of residual structure as opposed to what is often observed at extreme pH or high temperature. The renaturation is then induced by dilution in an adequate GdnHCl-free buffer and in many cases that have been described, the renatured protein regains the properties characterizing the native state as judged from the activity, the immunological reactivity and a variety of physico-chemical criteria. The pH, ionic strength, temperature, protein concentration and salts affect the yield of renaturation and the optimal conditions have to be determined for each protein (for review see [2]). Extensive studies on the renaturation of proteins demonstrate that the folding process proceeds through several intermediates becoming more and more organized as they are closer to the native state (for review see [3–6]). In this field, one of the most extensively characterized systems is the β_2-subunit of *Escherichia coli* tryptophan synthase. Previous reports from this laboratory described the existence of 6 intermediates on the folding pathway [7–11]. According to this result, β-chains renature at 12°C according to the following minimal

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Abbreviations: β_2, the β_2-subunit of *E. coli* tryptophan synthase (L-serine hydrolyase (adding indole) EC 4.2.1.20); I-AEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; pyridoxal-P, pyridoxal-5'-phosphate; GdnHCl, guanidinium chloride; β_2-AEDANS, β_2 labelled with I-AEDANS on cysteine 170; reduced holo-β_2, β_2 carrying the sodium borohydride reduced Schiff base between pyridoxal-P and the ϵ-amino group of lysine 87

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scheme:

$$\beta^{D}\text{-GdnHCl} \xrightarrow[\geq 0.07\ s^{-1}]{} \beta^{IR} \xrightarrow[\neq 0.02\ s^{-1}]{} \beta^{I1} \xrightarrow[0.008\ s^{-1}]{} \beta^{I2} \xrightarrow[0.001\ s^{-1}]{} \beta^{N'} \xrightarrow[\text{very rapid}]{} \beta_2^{N'} \xrightarrow[0.002\ s^{-1}]{} \beta_2^{N'} \xrightarrow[\text{rate limiting}]{} \beta_2^{native}$$

It can be seen that the rate constants of β^{IR} and β^{I1} appearances are too rapid to be conveniently measured by conventional manual mixing methods, even at 12°C where the folding process is already slowed down as compared to 37°C (the physiological temperature). To investigate in more details the kinetics of those two reactions, we tried to perform experiments in a multi-mixing stopped-flow apparatus. Some experimental artefacts due to the density differences existing between the 4 M GdnHCl-denatured protein solution (d^{20} = 1.114) and the phosphate buffer usually used for the renaturation of β chains (d^{20} = 1.014) led us to look for a high density renaturation buffer. Saccharose, or glycerol could be added to the buffer. For instance, a 16.5 g/l saccharose solution in a phosphate buffer (d^{20} = 1.086) compensates well enough the density of a 4 M GdnHCl solution but does not avoid other artefacts related to the very high viscosity of the final solution at low temperature. Furthermore, substances like saccharose or glycerol are known [12,13] to interfere with the structure of water, and hence with the hydrophobic interactions involved in the stability of the native proteins and folding intermediates. They thus should be avoided. In this report we show that the use of a phosphate buffer prepared in 90% heavy water (d^{20} = 1.110) avoids both artefacts described before and does not affect the functional properties of the renatured protein. The efficiency of heavy water phosphate buffer is illustrated by stopped-flow measurements of the rate constant of β^{I1} appearance performed at 12°C, by following the fluorescence signal reflecting this conformational change during the folding of β-chains denatured in 4 M GdnHCl.

2. MATERIALS AND METHODS

2.1. *Materials*

Fluoresceine-5-maleimide, I-AEDANS and pyridoxal-P were obtained from Sigma, ultrapure GdnHCl came from Schwarz-Mann, 98% D_2O was supplied by the Commissariat à l'Energie Atomique, Service des Molécules Marquées, CEN Saclay, 91191 Gif-sur-Yvette, France. All other chemicals were reagent grade. Buffer A: 100 mM potassium phosphate; 2 mM EDTA; 2 mM 2-mercaptoethanol; pH 7.8. Buffer B: buffer A in 90% D_2O final, pH 7.85 (given without any correction). Buffer C: 100 mM potassium phosphate; 100 mM L-serine; 0.05 mM pyridoxal-P; 2 mM 2-mercaptoethanol; pH 7.8. Buffer D: 4 M GdnHCl in buffer A.

2.2. *Methods*

The apo (i.e. free of pyridoxal-P) and holo (i.e. saturated with pyridoxal-P) forms of β_2 were prepared as described earlier [14]. Reduced holo-β_2 was obtained by sodium borohydride reduction of holo-β_2 [15]. β_2-AEDANS was prepared as described by Zetina and Goldberg [8].

The activity of β_2 was measured in the presence of the α-subunit as described by Faeder and Hammes [16]. The protein concentration was determined spectrophotometrically by using the specific absorbance reported by Högberg-Raibaud and Goldberg [14].

The denaturation was performed by diluting 2-fold native protein in a 2-fold concentrated buffer D and incubated 14 h at 4°C. Renaturation was initiated by a 30–50-fold dilution in buffer A or in buffer B by a vigorous manual mixing (vortex) or by using the Bio-logic (38130 Echirolles, France) stopped-flow SFM-3. The mechanical subsystem of the SFM-3 consisting of three syringes, two mixers, an ageing loop of 50 μl and an observation chamber, is enclosed in a water jacket connected to a circulating water bath thermostated at 12°C. The SFM-3 syringes are controlled by a Bio-logic MPS-5 microprocessor power-supply. The data on the kinetics were accumulated and analysed with a Tandon microcomputer interfaced to the SFM-3 module using the Bio-logic Bio-Kine hardware and software package. Monochromatic excitation light ($\lambda_{excit.}$ = 280 nm or 336 nm) was used and the fluorescence emission was recorded through a 350 nm or 450 nm high pass filter.

The standard protocol used to measure the yield of renaturation was as follows: denatured β-chains in buffer D were renatured by dilution in buffer A or in buffer B and incubated 2 h at 12°C. After centrifugation, 100 μl of a 10-fold concentrated buffer C were added to 900 μl of each supernatant. Then the samples were incubated 30 min at room temperature. The fluorescence of the 'Aqua complex', a fluorescent ternary complex between β_2, L-serine and pyridoxal-5'-phosphate [17] was then measured at 510 nm with a Perkin Elmer LS5-B spectrofluorimeter (excitation at 440 nm). It was verified that the fluorescence intensity of the Aqua complex per mg of native β_2 remains constant, even after a 30 min incubation in buffer C at room temperature, showing that hydrolysis of L-serine by the β_2-subunit taking place in these conditions does not affect the measurement.

3. RESULTS

3.1. *Recovery of functional properties after renaturation in a D_2O-phosphate buffer*

It was first checked that D_2O does not drastically

affect the overall refolding of β_2: native β_2 is able to interact specifically with its coenzyme, pyridoxal-5'-phosphate, and L-serine to form a fluorescent ternary complex, called the Aqua complex [17]. The binding of the two ligands can thus be easily monitored and it was used as a probe of the protein conformation. It should be noted however that, for native β_2, the fluorescence of the Aqua complex is 1.5-fold higher in buffer B than in buffer A. Denaturation and renaturation were performed as described in section 2.2. The fluorescence of the ternary complex was measured for each sample (R) and compared to the signal obtained for native β_2 at the same concentration and incubated in the same conditions (N). Fig.1 shows the variation of the renaturation yield (R/N) as a function of the protein concentration. The yield of renaturation decreases when the protein concentration increases in accord with the known tendencies of β_2-subunit to aggregate during its renaturation at high concentration [18] and it can be seen that no significant difference exists for β_2 renatured in a D_2O-phosphate buffer and for β_2 renatured in a H_2O-phosphate buffer.

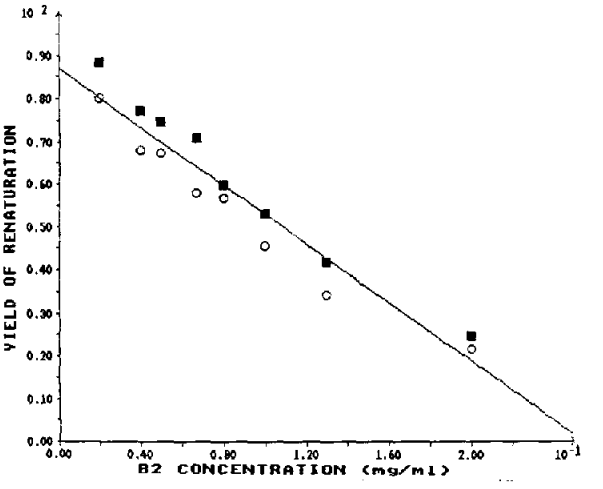

Fig.1. Protein concentration dependence of the yield of renaturation of β_2. β_2 was denatured in 4 M GdnHCl, at +4°C, and renatured by dilution in buffer A (■), or in buffer B containing 90% D_2O (○) followed by an incubation for 2 h at 12°C. After centrifugation, 900 μl of each sample were added to 100 μl of 1 M potassium phosphate, 1 M L-serine, 0.5 mM pyridoxal-5'-phosphate, 2 mM 2-mercaptoethanol and then incubated 30 min at room temperature. The fluorescence of the ternary complex formed ($\lambda_{excit.}$ = 440 nm, $\lambda_{emiss.}$ = 510 nm) was measured for each sample (R) and compared to that obtained for native β_2 incubated in the same conditions (N). The yield of renaturation was calculated as $100 \times R/N$.

3.2. *Determination of mixing conditions*

β_2 was shown to be completely denatured in 4 M GdnHCl, and to be in a native conformation at 0.1 M GdnHCl according to its circular dichroism spectrum, to its sedimentation coefficient and to the fluorescence of its tryptophan residues [7]. Furthermore, 0.1 M GdnHCl does not seem to affect significantly the kinetics of refolding of β_2 [8]. We therefore chose to study the refolding of β_2 by reducing the GdnHCl concentration from 4 M to 0.1 M (or below) in a stopped-flow. It was first necessary to set up the mixing conditions using the SFM-3 stopped-flow module. The system was equipped with 2 standard 18 ml syringes, and a small 5 ml syringe. The flow rate was limited at one and the same time by the minimum duration of the drive impulses and by the volume injected per step. The 4 M GdnHCl solution was contained in the small syringe and the two standard syringes were filled up with the D_2O-phosphate buffer. A solution of fluorescein-5-maleimide (2×10^{-5} M), prepared in a 4 M GdnHCl phosphate buffer, was used to calibrate the system ($\lambda_{excit.}$ = 490 nm; 530 nm high pass filter). First, the fluorescein-5-maleimide was diluted in the D_2O-phosphate buffer through the first mixer and part of the solution thus obtained was stored in the 50 μl ageing loop. The volume injected was largely sufficient to fill up the 62 μl observation cuvette. The intensity of the fluorescence obtained was compared to the initial fluorescence of the fluorescein solution. Second, 20 μl of the content of the ageing loop were pushed out of the ageing loop, using the small syringe, and simultaneously diluted, through the second mixer with the D_2O-phosphate buffer contained in the third syringe. The final fluorescence intensity was compared to the one obtained after the first mixing. It must be pointed out here that the observed dilutions are not exactly equal to the calculated ones. For example, the procedure described in the legend of fig.2 gives a $5.5 \times 6.5 = 35.7 \pm 5$ dilution yield. Because this does not occur with an initial fluorescent solution prepared in a D_2O-phosphate buffer, rather than in 4 M GdnHCl this artefact is likely to be due to the high viscosity of the 4 M GdnHCl solution.

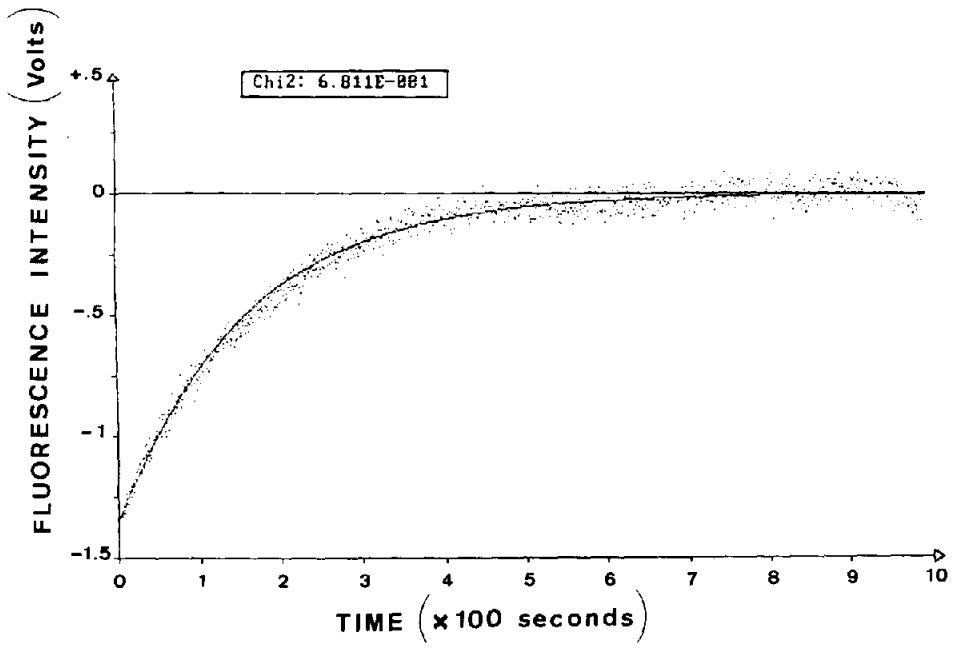

Fig.2. Kinetics of appearance of the tryptophan to AEDANS energy transfer in β_2. The 4 M GdnHCl denatured β-chains (1 mg/ml) contained in the small syringe (20 μl of channel 2) were first diluted in D_2O-phosphate buffer (230 μl of channel 1). Then 20 μl of the resulting mixture stored in the 50 μl ageing loop were diluted in the same buffer (20 μl of channel 2 and 180 μl of channel 3). This multi-mixing protocol gave a 5.5 × 6.5 = 35.7 ± 5 dilution of β-chains. The injection time was 50 ms for each mixing phase. The data acquisition started at the end of the second injection phase. The increase of the fluorescence ($\lambda_{excit.}$ = 280 nm, 450 nm high pass filter) was recorded as a function of time. The figure also shows (solid line) the fit obtained for a single exponential reaction.

3.3. *Renaturation of dansylated Apoβ_2 in a deuterated phosphate buffer*

Cysteine 170 of the β_2-subunit is the single one among the 5 per β-chain to be reactive to sulfhydryl reagents in the native holoenzyme [19]. It was shown that during the folding of β-chains, the tryptophan 177 comes close to a dansyl group (AEDANS) covalently linked to cysteine 170 giving rise to a fluorescence energy transfer from the tryptophan ($\lambda_{excit.}$ = 295 nm) towards the AEDANS ($\lambda_{emis.}$ = 470 nm) [10]. We first tested if renaturation on GdnHCl-denatured β-AEDANS can be followed in the stopped-flow apparatus and whether or not D_2O affects the kinetics of appearance of the fluorescence transfer signal. The large dilution required to reduce the guanidine concentration from 4 M to about 0.1 M was obtained by two successive mixings in buffer B at 12°C as previously described (see section 3.2). Fig.2 shows the kinetics obtained for the appearance of the tryptophan to dansyl fluorescence transfer. Analysis of the data gave a good fitting with a single exponential process (fig.2). The rate constant obtained did not vary with the protein concentration (10–50 μg/ml). This reaction therefore appears to obey first order kinetics. The value obtained for the rate constant (k = 0.008 ± 0.001 s^{-1}) is similar to the one obtained by hand mixing for β-AEDANS renaturation in the usual phosphate buffer (0.008 ± 0.001 s^{-1}). Thus the presence of deuterium oxide in the renaturation buffer does not affect the kinetics of the folding step giving rise to the tryptophan 177 to the AEDANS-Cysteine 170 fluorescence energy transfer. Furthermore, at the end of the reaction, the fluorescence signal was very stable, and no mixing artefact took place in the observation chamber as opposed to experiments where the renaturation was performed in the stopped-flow using the usual phosphate buffer.

3.4. *Renaturation of reduced holo-β_2*

It was reported previously that during the folding of sodium borohydride-reduced holo-β_2,

tryptophan 177 comes close enough to lysine 87 to give rise to a fluorescence energy transfer between the single tryptophan of the β-chain and the reduced Schiff base of pyridoxal-5'-phosphate covalently linked to the lysine 87 at the active site [10]. But the rate constant of this folding step was too fast to be measured at 12°C by hand mixing and was only estimated by extrapolation at 12°C of an Arrhenius plot obtained from measurements at low (2–6.5°C) temperatures. The setting up of conditions for the renaturation in D_2O-phosphate in the stopped-flow apparatus, allowed us to study the kinetics of formation of this intermediate during the folding of β-chains at 12°C. Fig.3 shows the increase of the fluorescence signal ($\lambda_{excit.}$ = 280 nm; 350 nm high pass filter) as a function of time. It was verified that the fluorescence of the Schiff base itself ($\lambda_{excit.}$ = 335 nm; 350 nm high pass filter) remained constant during the same experiment. This shows that there is no change in the quantum yield of the reduced Schiff base during the renaturation of β_2, and that the signal observed indeed corresponds to a fluorescence energy transfer. The rate constant obtained by the data analysis program was found to be independent of the protein concentration (10–46 μg/ml) and equal to $k = 0.014 \pm 0.002\ s^{-1}$ at 12°C (fig.3), a value compatible with that previously estimated by extrapolation of the Arrhenius plot ($k = 0.02 \pm 0.005\ s^{-1}$).

4. DISCUSSION

The results described above show that two major technical problems which have often precluded the use of a commercially available stopped-flow for studying the renaturation of guanidine or urea unfolded proteins can be easily overcome. Indeed large dilution factors (here about 40) can be reached by use of a three-syringe, double-mixer stopped-flow apparatus. And mixing artefacts as well as lack of signal stability, due to the density differences between the GdnHCl solution and the renaturation buffer, can be eliminated even in a vertical stopped-flow by use of a high density renaturation buffer prepared in heavy water (D_2O). Thus, 90% D_2O permits compensation of the density of 4 M GdnHCl.

The efficiency of this experimental approach is demonstrated by measurements of the rate of ap-

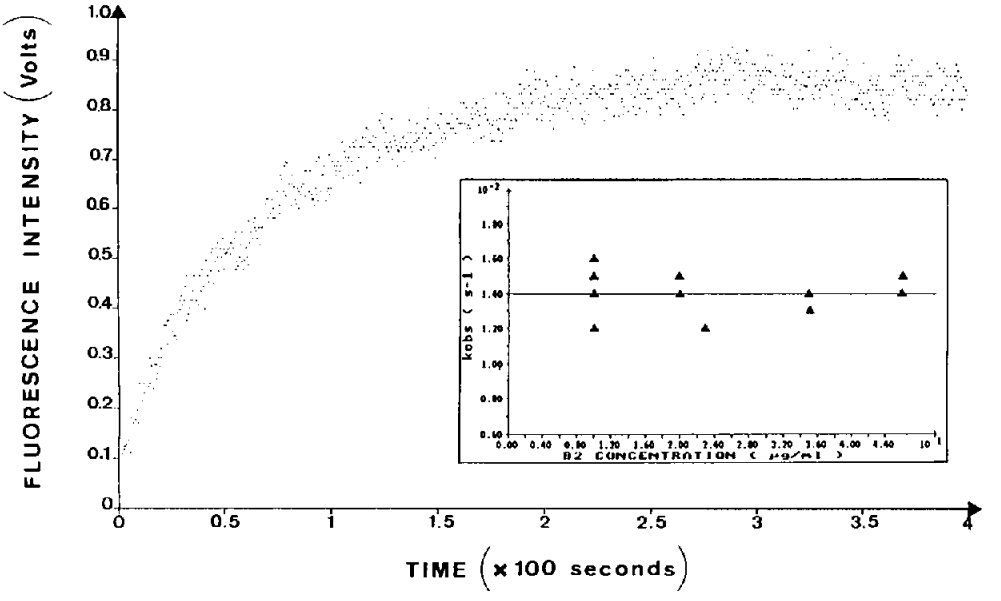

Fig.3. Kinetics of appearance of the tryptophan to reduced Schiff base energy transfer in β_2. Reduced holo-β chains (0.35 mg/ml), denatured in 4 M GdnHCl, were diluted using a multi-mixing protocol similar to the one described in fig.2. The increase of the fluorescence ($\lambda_{excit.}$ = 280 nm; 350 nm high pass filter) was recorded as a function of time. (Inset) Variation of the rate constants obtained for a single exponential reaction (calculated by the data analysis program) as a function of the protein concentration.

pearance of some intermediates during the refolding of the GdnHCl-denatured β_2-subunit of *E. coli* tryptophan synthase. Firstly, the appearance of an early intermediate (characterized by a fluorescence transfer from tryptophan 177 to the reduced Schiff base between pyridoxal-P and lysine 87) could easily be observed at 12°C in the stopped-flow apparatus, while at that temperature it was too fast to be followed after hand mixing [10]. This illustrates the efficiency of the double-mixer stopped-flow for rapidly achieving large dilutions. While no attempt was made in this study to minimize the dead-time of the stopped-flow, it was well below 100 ms in our experiments. Secondly, it is shown that the kinetics of appearance of a second intermediate (characterized by a fluorescence transfer from tryptophan 177 to AEDANS-labeled cysteine 170) observed in the stopped-flow are identical to the kinetics observed by conventional hand mixing methods.

Since these rather slow ($k = 0.008\ s^{-1}$) reactions gave rise to smooth curves which fit single exponentials very well, it can be concluded that mixing artefacts and 'back-diffusion' of reagents due to density differences have efficiently been abolished by the use of D_2O. Furthermore, the identity of the kinetics observed in D_2O-phosphate (in the stopped-flow) and in H_2O-phosphate (hand mixing) as well as the lack of effect of D_2O on the renaturation yield (fig.1), demonstrate that D_2O does not drastically alter the folding process. This conclusion is also supported by the similarity of the rate constants obtained for the early intermediate either directly at 12°C in D_2O ($k = 0.014 \pm 0.002\ s^{-1}$), or by extrapolation at 12°C of the Arrhenius plot of rate constants at low temperature in H_2O ($k = 0.020 \pm 0.005\ s^{-1}$). Thus, D_2O seems not to introduce a significant bias in folding kinetics.

It should however be reminded that D_2O has been reported to sometimes have some effect on protein stability [20,21], or protein activity [22]. Therefore, though D_2O or ^3H_2O have already been used to investigate early events in protein folding [23–25] one should bear in mind that isotope effects still might slightly alter the kinetics of very rapid steps in protein folding.

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