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Sequential mechanism of refolding of carbonic anhydrase B

Gennady V. Semisotnov, Natalya A. Rodionova, Victor P. Kutyshenko+, Bernd Ebert*, Jürgen Blanck* and Oleg B. Ptitsyn

Institute of Protein Research, +Institute of Biological Physics, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR and **Central Institute of Molecular Biology, Academy of Sciences of the GDR, 1115 Berlin, GDR*

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The kinetics of refolding of bovine carbonic anhydrase B was studied by a variety of methods over a wide range of times (from milliseconds to hours). It has been shown that protein refolding proceeds through three stages. At the first stage ($t_{1/2} \approx 0.03$ s) hydrophobic clusters and a compact state of the chain are formed. At the second stage ($t_{1/2} \approx 140$ s) hydrophobic clusters are desolvated and the rigid native-like hydrophobic core is formed. At the third stage ($t_{1/2} \approx 600$ s) the native active protein is formed.

Carbonic anhydrase B; Refolding kinetics; Kinetic intermediate; Hydrophobic probe; ^{1}H-NMR; ESR; Energy transfer; Fluorescence

1. INTRODUCTION

The kinetics of protein folding in vitro from a completely unfolded state is usually not a monophasic process [1]. This can be due either to the heterogeneity of the unfolded state (for example, as a result of *cis-trans*-proline isomerization [2]) or to the existence of a sequential mechanism of protein folding [3]. The choice between these possibilities can be made by studying the kinetics of protein folding by different methods which give information on the structural characteristics of a protein.

In particular, it has been shown that refolding of carbonic anhydrase B proceeds through an intermediate state [4–7] which has been identified as a 'molten globule' state [7]. However, the refolding kinetics was studied at time intervals >100 s, therefore the formation of early kinetic intermediates could not be followed.

Correspondence address: G.V. Semisotnov, Institute of Protein Research, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR

Abbreviations: $t_{1/2}$, half-time of the process; CD, circular dichroism; ANS, 8-anilino-1-naphthalenesulfonate

Here, we studied the kinetics of refolding of bovine carbonic anhydrase B over a broad time range to characterize kinetic intermediates and to measure the times of their formation.

2. MATERIALS AND METHODS

Carbonic anhydrase B was isolated from bovine erythrocytes according to [8]. The protein was labelled in Tris-HCl buffer (pH 8.5) in the presence of a 10–20-fold label excess over 12 h. Free labels were removed by dialysis and gel filtration on G-25. The degree of labelling was 3 labels per protein molecule. The labelled protein retained its esterase activity [8] and its main physico-chemical properties.

ESR spectra and the kinetics of their changes were measured with a Varian E3 ESR spectrometer (USA) and an SFA-1 stopped-flow attachment (GDR) [9]. Fluorescence, absorption and CD spectra were measured with an Aminco SPF-1000^{CS} spectrofluorimeter (USA), an M-40 spectrophotometer (GDR) and a Jobin Iron mark IIIS spectropolarimeter (France). Fast changes of the fluorescence were checked using a Durrum stop-flow fluorimeter (USA). ^{1}H-NMR spectra were

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measured with a Bruker WM400 NMR spectrometer (FRG) at 400 MHz. The interval between successive accumulations was 1 s, and the duration of a measuring impulse was 8 μs. The number of accumulations was constant and equal to 120.

Protein refolding was initiated by mixing the protein solution in 8.5 M urea with the buffer to a final urea concentration of 4.1–4.3 M (at 23°C). The slow refolding kinetics was studied by manual mixing (dead-time 10–120 s) and the fast one by the stopped-flow technique (dead-time 1–5 ms). Protein concentrations were from 4–5 mg/ml (for ^{1}H-NMR) to 0.04–0.08 mg/ml (for the fluorescence study).

3. RESULTS

Fig.1 shows the kinetics of carbonic anhydrase B compactization monitored by (a) the increase in spin-label immobilization by the protein matrix and (b) the increase in energy transfer from tryptophan residues to dansyl labels. Both curves are well approximated by one exponent with practically the same rate constant ($\sim 20\ s^{-1}$).

Fig.2 presents the increase in fluorescence of a hydrophobic probe (ANS) reflecting its adsorption by the polypeptide chain during protein refolding. It has been shown recently (Semisotnov, G.V. and Rodionova, N.A., unpublished) that ANS has a strong affinity for a solvated hydrophobic core formed in the molten globule state (carbonic anhydrase B and α-lactalbumins at acid pH and an intermediate concentration of guanidine hydrochloride). However, it binds weakly both with unfolded chains and with native proteins. It has also been shown that ANS binding with a polypeptide chain by itself proceeds faster than in 1 ms. Therefore, we suggest that the increase in fluorescence intensity of ANS during refolding (fig.2) reflects the kinetics of formation of hydrophobic clusters.

Fig.3 shows the decrease in fluorescence intensity of ANS reflecting its desorption from a protein. This process is well described by one exponent with a rate constant of $\sim 0.005\ s^{-1}$. The points in fig.3 present the increase in area under the high-field signals in the ^{1}H-NMR spectrum (from 0.7 to -0.6 ppm). These points are well described by the calculated exponent (dotted line) with the same rate constant.

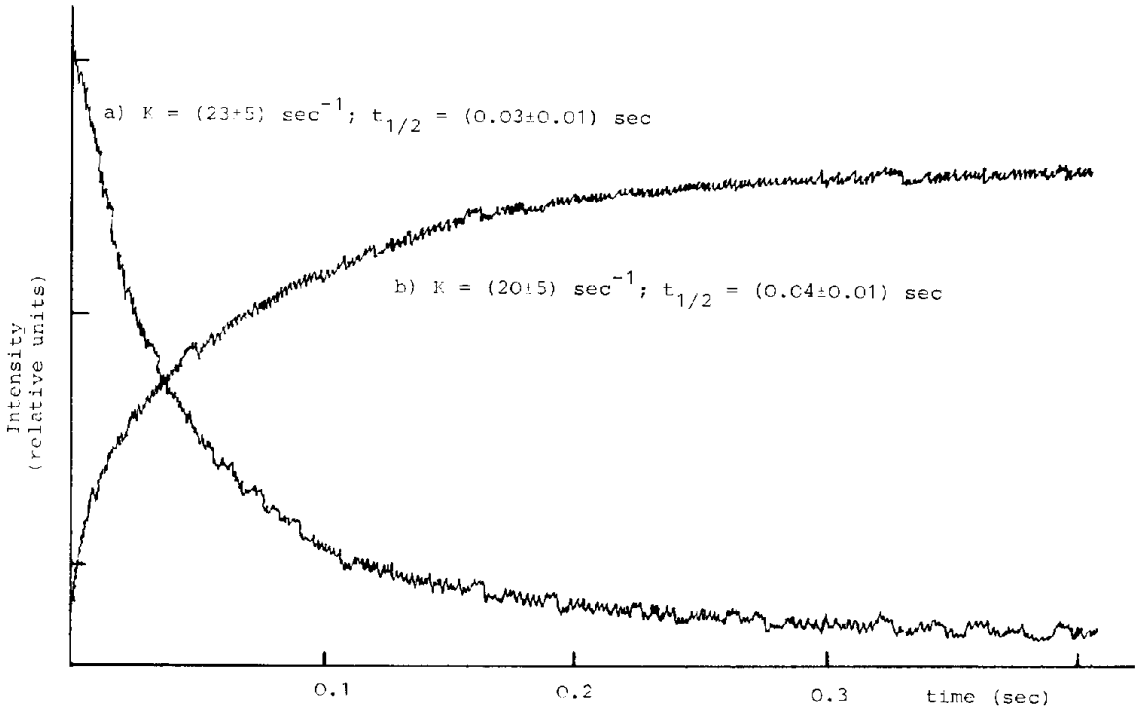

Fig.1. Decrease in intensity of the spin-label spectrum (a) and increase in intensity of the dansyl label fluorescence (λ_{em} = 500 nm) upon excitation of tryptophan residues (λ_{ex} = 290 nm) (b) in the refolding process.

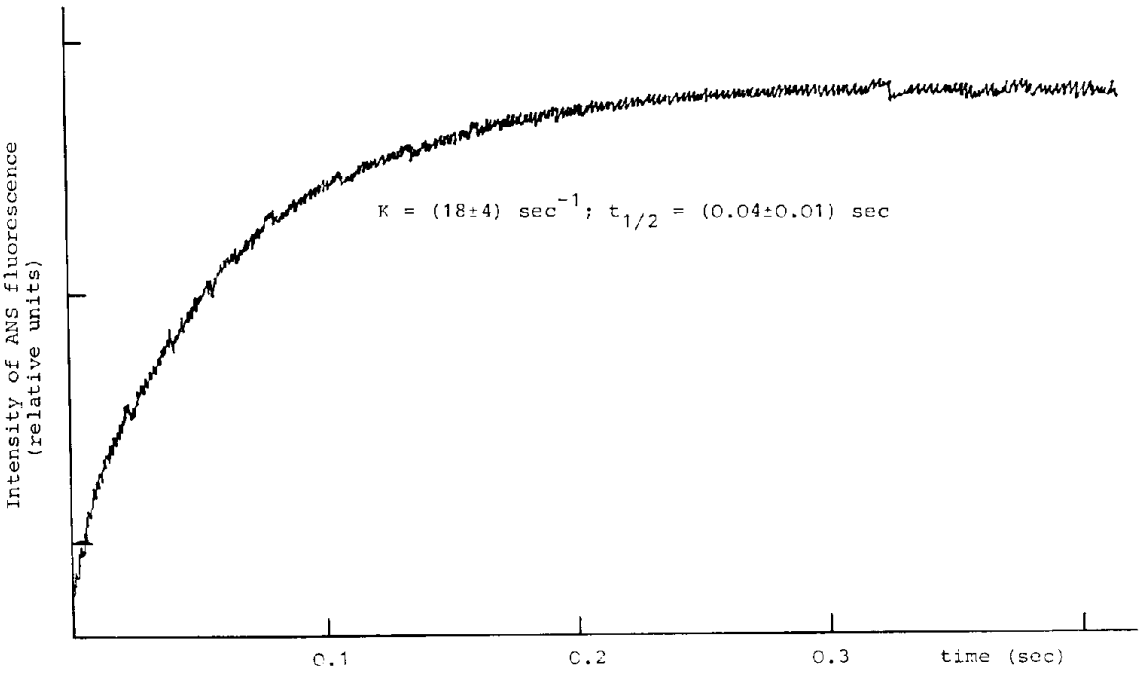

Fig.2. Increase in ANS fluorescence intensity in the refolding process. The ratio of molar concentrations ANS/protein = 10. λ_{ex} = 390 nm, λ_{em} = 500 nm.

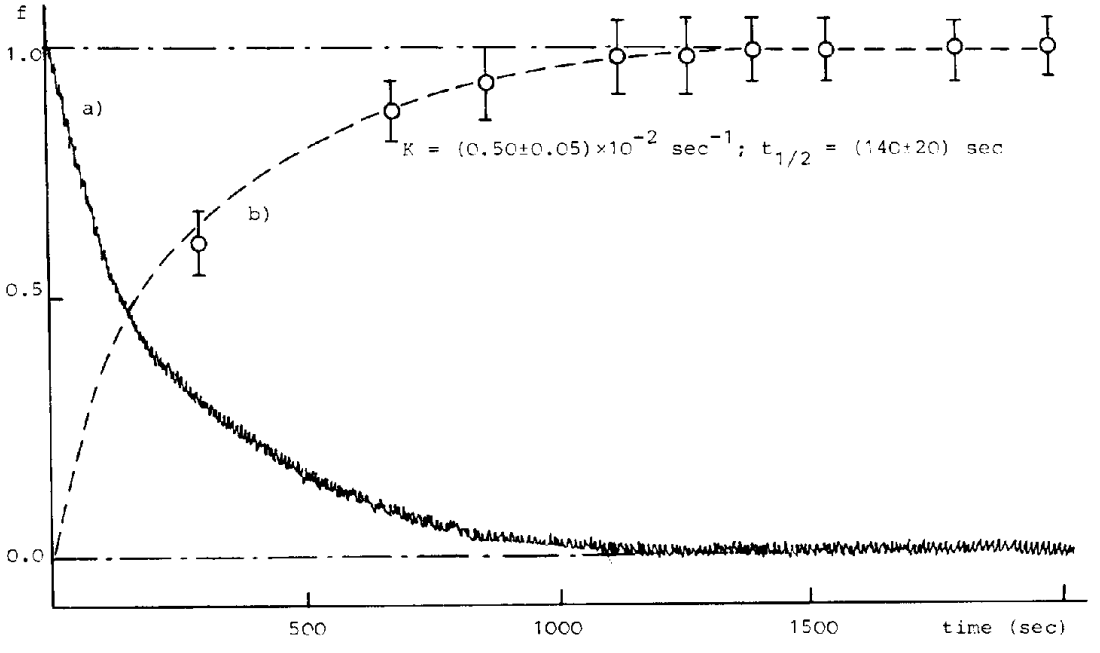

Fig.3. Decrease in ANS fluorescence intensity (a) and increase in area under the high-field signals (from 0.7 to −0.6 ppm) of the ^{1}H-NMR spectrum (b) in the refolding process. The curves are normalized to the parts (f) of the unfolded state for curve (a) and of the refolded state for curve (b).

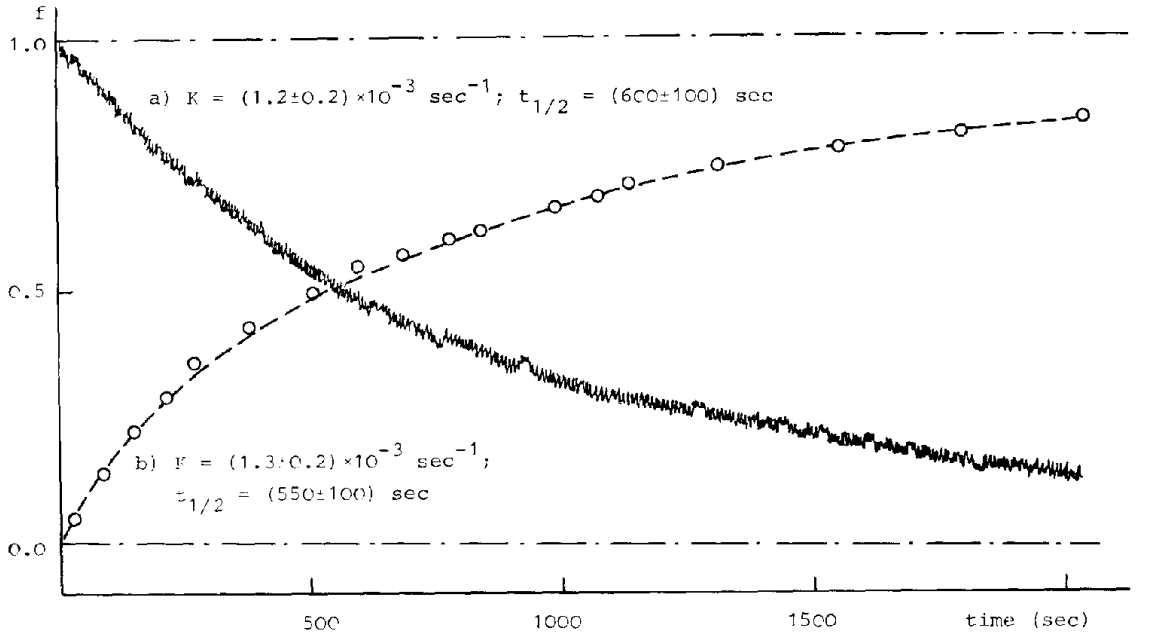

Fig.4. Restoration of ellipticity at 270 nm (a) and of esterase activity (b) in the refolding process. The curves are normalized as in fig.3.

Fig.4 presents the increase in ellipticity of the aromatic group absorption $[\theta]_{270}$ and the increase in esterase activity. Both curves are well approximated by one exponent with practically the same rate constants of ~ 0.001 s^{-1}.

Kinetic curves of the increase in aromatic group absorption (not shown) can be resolved into two exponents with comparable amplitudes and rate constants of 0.006 ± 0.001 and 0.0010 ± 0.0002 s^{-1}.

4. DISCUSSION

Three well resolved stages can thus be distinguished in the kinetics of carbonic anhydrase refolding.

The first stage with $t_{1/2} \approx$ (0.03–0.04) s is connected with compactization of the protein chain as is evident from the increase in energy transfer and from the increase in spin-label immobilization (fig.1). It is likely that these processes reflect the global (rather than local) changes of the protein structure as: (i) the labels reacted with numerous amino groups of the protein; (ii) the degree of labeling was three labels per protein molecule; (iii) bovine carbonic anhydrase B has eight tryptophan residues uniformly distributed along the chain. This means that the labels are statistically distributed along the protein chain and therefore must reflect the global changes of the molecule.

The increase in hydrophobic probe affinity which takes place simultaneously with the compactization process (fig.2) suggests that hydrophobic clusters are formed at the same stage. CD studies by manual mixing have shown [6,7] that the secondary structure of carbonic anhydrase is restored in less than 100 s. CD measurements with the stopped-flow technique have not yet been performed. However, the formation of hydrophobic clusters and a compact state at the first stage of refolding (as well as the absence of other stages at times lower than 100 s suggests the presence of the secondary structure already in the first kinetic intermediate.

The second stage with $t_{1/2} \approx (140 \pm 20)$ s is three orders of magnitude slower than the first. It is characterized by desolvation of hydrophobic clusters (desorption of the previously bound ANS) and the formation of a rigid native-like hydrophobic core (increase in area under high-field resonances of the ^{1}H-NMR spectrum) (see fig.3). The high-field ^{1}H-NMR spectrum reflects the in-

teraction of aliphatic and aromatic side chain groups in the rigid tertiary structure and is usually a sort of 'fingerprint' of the protein [10]. It strongly suggests that the protein acquires its specific folding pattern at least at the second stage. The time of this stage is close to that of *cis-trans*-proline isomerization and therefore this stage may be proline-dependent.

The third stage with $t_{1/2}$ = (600 ± 100) s is characterized by the restoration of protein enzymatic activity (fig.4a) and of the native environment of side groups (fig.4b and the ^{1}H-NMR spectrum, not shown). This process is too long to be connected directly with proline isomerization. As the secondary structure and the native-like hydrophobic core are already formed, we suggest that at this stage loops and other irregular chain regions acquire their native structures and surface side groups become fixed.

Thus the sequential mechanism of the refolding of carbonic anhydrase B can be represented as follows:

		hydrophobic clusters,	secondary structure,		
	0.03_5 s	compactness	140 s rigid	600 s	native
coil	→	→	→ hydrophobic	→	protein
		secondary structure?	core		
		folding pattern?	folding pattern?		

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