ON THE EFFECT OF DIVALENT CATIONS AND PROTEIN CONCENTRATION UPON RENATURATION OF β-GALACTOSIDASE FROM E. COLI.

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Received February 25, 1969

It is widely accepted today that the *native* state of globular proteins corresponds to the thermodynamically most stable conformation of the molecule, in water solution, as dictated by its aminoacid sequence. The main experimental support for this belief are those instances where spontaneous renaturation of proteins, from a state of complete or partial unfolding, has been obtained (1).

While there are now quite a few examples of such successful renaturation experiments, there are presumably many more which have met with no success. We say *presumably* because most of these negative experiments never get into print. Should we then believe that there are two kinds of proteins : those where the native state does correspond to a trough in potential, and therefore will refold spontaneously, and those whose native conformation may be kinetically rather than thermodynamically stable, and therefore cannot spontaneously refold correctly ? Few biochemists would be ready to accept this unpleasant conclusion, even though it is implicit in some proposals recently made by Levinthal (2), concerning the rules of polypeptide folding.

Yet, the reasons why so many proteins will not efficiently anneal after being denatured remain obscure. In the present

paper, we wish to call attention to certain conditions and agents which play a decisive role in allowing the renaturation of β-galactosidase (from *E. coli*) following treatment with urea, guanidine or heat.

The enzyme β-galactosidase (MW : 540,000) is a tetramer, made up of four protomers, each involving a single polypeptide chain (MW : 135,000). It was first found by Zipser (3) that

T A B L E I

RECOVERY OF β-GALACTOSIDASE AFTER DIFFERENT TREATMENTS

Treatment	U/ml β-galactosidase	% recovery
None	270,000	
Dialysis against 8 M urea then TVNS	280,000	104
1 min. at 100° C	0	0
1 min. at 100° C, dialysis against 8 M urea, then TVNS	267,000	99
Dialysis against 6 M guanidine HCl, then TVNS	2,050	0.75
Dialysis against 6 M guanidine, then 8 M urea, then TVNS	202,000	75

Pure crystalline β-galactosidase was dialyzed for 24 hours against TVNS buffer (2.10^{-2}M Tris, 10^{-2}M EDTA, 10^{-2}M NaCl, 10^{-1}M β-mercaptoethanol, pH 7.2). Urea and guanidine-HCl were dissolved in the same buffer. The urea and guanidine treatments consist in dialyzing the β-galactosidase solution against these agents for 8 hours, followed by a 16 hours dialysis against TVNS buffer. The β-galactosidase activity was measured after incubating the different samples for 8 hours at 28°C in PM_2 buffer (5). For definition of enzyme units and method of estimation : see (5).

active enzyme could be recovered in good yield after treatment with 8 M urea. Perrin and Monod later showed that fairly good recovery (12 to 25%) could be obtained also after treatment by heat or 6 M guanidine (4).

The results of similar experiments are shown in Table I. The only significant difference between these experiments and those mentioned above, was the use of a buffer (TVNS) containing 10^{-2}M EDTA, and no Mg^{++}. Since the recoveries (virtually 100%

T A B L E II

EFFECTS OF DIVALENT CATIONS ON THE RECOVERY OF β-GALACTOSIDASE

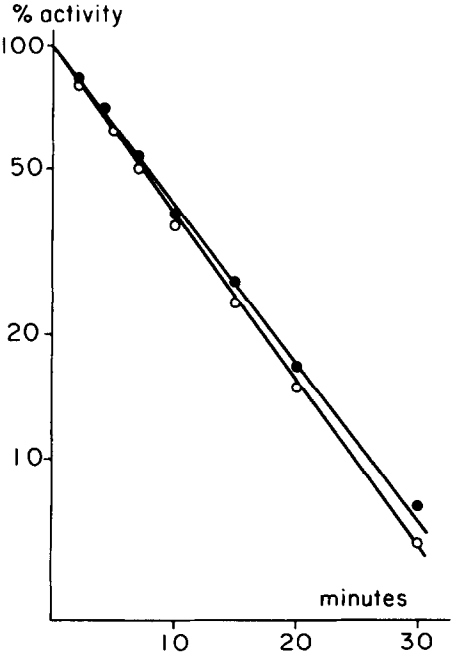
Experiment	Cation	Concentration (M)	U/ml β-galactosidase after dialysis	% recovery
1	-	-	1,400,000	100
	Mg^{++}	2.10^{-4}	1,150,000	82
	"	5.10^{-3}	1,000,000	72
	"	10^{-2}	33	0.003
	"	3.10^{-2}	0	0
	"	10^{-1}	146	0.01
2	-	-	94,500	100
	Mg^{++}	2.10^{-2}	0	0
	Mn^{++}	2.10^{-2}	0	0
	Zn^{++}	2.10^{-2}	0	0
	Ca^{++}	2.10^{-2}	0	0

A β-galactosidase solution in TVNS buffer was dialyzed against 8 M urea solution as indicated in Table I. The elimination of the urea was performed by dialysis against TVNS buffer with or without addition of divalent cations as indicated in the Table.

after urea alone, 75% after guanidine, 100% after one minute at 100°C) were very high, we suspected that the presence of Mg^{++} might prevent renaturation. This is clearly shown in the experiment summarized in Table II : at/or above 10^{-2} Mg^{++}, no recovery of activity is observed (as against 100% in its absence), while, we should note, a precipitate forms during dialysis of urea.

Similar results are obtained with other divalent cations (Zn^{++}, Ca^{++}, and Mn^{++} : see Table II).

Recovery of activity, even complete, after urea treatment, may not be considered a sufficient criterion that the renatured



Heat inactivation of β-galactosidase. Pure crystalline β-galactosidase dissolved in TVNS buffer was dialyzed against 8 M urea solution, as indicated in Table I. After elimination of urea by dialysis against TVNS buffer, the enzyme solution was diluted 20 times with PM_2 buffer. Heat inactivation was performed at 57.5° C in the presence of PM_2 buffer.

●——● Untreated β-galactosidase

o——o Urea treated β-galactosidase

enzyme has truly returned to its native state. A more stringent test is shown in Fig. 1. It is seen that the kinetics of heat denaturation of the native and urea-treated renatured enzymes are virtually the same.

Two other facts should also be mentioned in connection with these observations :

a. The enzyme renatured by dialysis in the absence of Mg^{++} is virtually inactive. Activity is instantaneously restored upon addition of Mg^{++}.

b. The renatured, inactive enzyme (before addition of Mg^{++}) exhibits an S of 16, in sucrose gradient, i.e. the same value as observed with the native tetrameric enzyme. Mg^{++} therefore appears not to be required for the formation of the tertiary or quaternary structures. It seems more likely to play exclusively a local role, at the active site.

Yet it must be asked whether the strong effect of Mg^{++} upon renaturation of β-galactosidase might be somehow related to the specific requirement of this ion for activity. We have observed however, that treatment of BSA under precisely the same conditions as above - 8 M urea in TVNS buffer, with or without added Mg^{++} - yields closely comparable results. In the presence of Mg^{++}, dialysis of the urea leads to the formation of a large precipitate, containing 95% of the protein, while in the absence of Mg^{++}, the protein remains entirely in solution.

While we have performed no experiments to test whether BSA in the latter case, was renatured to its *native* conformation, it is clear that this cannot occur in the presence of Mg^{++}.

The simplest interpretation of these observations appears to be that, in presence of Mg^{++} and other divalent cations, multiple interactions occur between peptide chains (presumably

through formation of metal chelates) *before* the chains start refolding into a water-soluble configuration. This interpretation implies that *correct refolding* is a monomolecular process, which is disfavored by the occurence of interactions between unfolded chains, the latter leading to illicit (water-soluble) associations.

This interpretation is supported by the results of an experiment (summarized in Table III) which attempts to account, in part, for the fact that renaturation by dialysis from 6 M guanidine is so very inefficient, as compared with the results obtained with urea. In this experiment, the per cent recovery of β-galactosidase activity was determined over a wide range of initial protein concentrations. The remarkable fact is that the

T A B L E III

RECOVERY OF β-GALACTOSIDASE AFTER GUANIDINE TREATMENT

β-galactosidase		U/ml after dialysis	% recovery
mg/ml	U/ml		
0.33	300,000	370	0.12
0.11	100,000	1,100	1.1
0.036	33,000	2,940	8.9
0.012	11,000	3,300	30
0.004	3,700	1,120	30

β-galactosidase solutions in TVNS buffer at different protein concentrations were dialyzed against 6 M guanidine HCl as indicated in Table I, followed by a 16 hours dialysis against TVNS buffer. The activities were measured as indicated in Table I.

per-cent recovery *increases* some 300 fold (reaching the respectable figure of 30%) as the protein concentration *decreases* down to 0.01 mg/ml.

This result suggests that during dialysis of guanidine, chain to chain interaction may occur *before* folding, thereby preventing correct monomolecular renaturation of individual chains. Presumably this effect is minimized where urea is concerned.

To summarize : the preliminary observations reported above show that two entirely different *pathways* may be followed by a solution of denatured protein during removal of the denaturating agent. At relatively high protein concentration and in the presence of certain ions, multiple interactions between chains may occur leading to an inactive precipitate. At lower concentrations of protein, and in the absence of these ions, refolding of individual peptide chains onto themselves is favored, leading to restoration of the native state.

One of these two states should, presumably, be considered as metastable with respect to the other. But it seems difficult to decide which of the two constitutes a *kinetic trap*. The observations certainly suggest that the native tertiary structure of a protein corresponds to the thermodynamically most stable state of its polypeptide chain(s) in *isolation :* whether this is also true of a *population* of molecules appears doubtful. Once folded into a compact soluble conformation, a kinetic barrier (well known to be high) will prevent illicit multiple association between chains. Preservation of the native state, in a solution of protein, may be due to this kinetic barrier, rather than to thermodynamic stability. In this restricted sense, it may be correct to say that an obligatory *pathway* (2) must be

followed in the formation of biologically correct tertiary and quaternary structures, and it may not be correct to assert that a *solution* of native protein is, or ever may be, in its thermodynamically most stable state.

Research in the Department of Cellular Biochemistry at the Pasteur Institute has been aided by grants from the U.S. National Institutes of Health, the "Centre National de la Recherche Scientifique", the "Délégation Générale à la Recherche Scientifique et Technique", and the "Commissariat à l'Energie Atomique".

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