

The invention concerns a process for the renaturation of proteins.

By renaturation of proteins in the scope of the present invention is to be understood the production or reobtaining of the normal biological function of proteins, whereby it is assumed that the natural secondary, tertiary and possibly quaternary structure is reobtained.

The problem of the renaturation of proteins is of great importance since biologically-active proteins are frequently denatured by the treatments carried out upon them, for example in the case of their separation from accompanying materials, and the interesting biological activity is hereby lost completely or partly. Renaturation is also of importance in the case of proteins produced synthetically or according to methods of gene technology since, in many cases, one hereby obtains only the correct primary structure, thus amino acid sequence, but the desired utilisation of the biological activity requires that a renaturation takes place with the formation of the natural secondary structure, tertiary structure and possibly also the quaternary structure.

From US-PS 4,530,787, it is already known to oxidize synthetic proteins, which contain reduced cysteine, with iodosobenzoate with reobtaining of their biological activity. Even if this method were to be of general applicability, it would only solve a part of the problem of the renaturation of proteins.

G. Zettlmeissl, R. Rudolph and R. Jaenicke, Biochemistry, Vol. 18 (1979), pages 5567 to 5575, have investigated the renaturation of an oligomeric enzyme, namely lactate dehydrogenase, with reobtaining of its tertiary structure and have thereby ascertained that in the case of such a renaturation, two competing

reactions take place, namely, the renaturation on the one hand and the formation of inactive aggregates on the other hand. For the achievement of good renaturation yields, care must thereby be taken that a limiting concentration of enzymes in the solution is not exceeded. However, a general process for the renaturation of proteins cannot be derived from this.

Therefore, it is the task of the invention to provide a process which, in general, makes possible an improvement of the renaturation of proteins.

According to the invention, this task is solved by a process for the renaturation of denatured proteins in solution in a renaturation buffer, which is characterised in that one prepares a solution of the protein to be renatured in the concentration in which, in the selected buffer, the highest yield of native renatured protein is obtained (critical concentration) and, after formation of the folding intermediate, adds in the amount necessary for the achievement.

The process is based upon the discovery that a highest possible yield of renatured protein can be achieved with a simultaneous maximum speed of renaturation when a definite critical concentration value of the denatured protein to be renatured in the solution is not exceeded, whereby this value is dependent upon the nature of the protein in question and the conditions under which the renaturation is carried out. It is thereby important that the relationship of two reactions competing with one another, namely, the undesired formation of inactive protein (e.g. aggregates) on the one hand and the formation of a folding intermediate on the other hand, which subsequently passes over into the final native renatured protein, is so displaced that only the smallest possible proportion of inactive protein can arise. For this purpose, it is necessary

to take into account the protein-specific and the condition-specific critical concentration of the protein to be renatured, the renaturation kinetics and the time up to the formation of the folding intermediate.

These three parameters important for the process can be readily determined. The critical concentration can be ascertained by means of a calibration curve which one obtains in that one uses different concentrations of denatured protein in the particularly selected renaturing buffer and thereby measures the end yield of renatured protein achieved in a predetermined period of time. The critical concentration corresponds to the value at which the highest yield (relative yield in % of the starting concentration) of native renatured protein is obtained.

The renaturing kinetics can also be determined according to known methods. There is thereby determined, at the maximum possible concentration, the time necessary for the conversion into renatured protein. The time up to the formation of the folding intermediate can finally be determined according to a whole series of different processes. A first method of determination consists in the measurement of the circular dichroism (CD). A wavelength is thereby expediently chosen at which a large signal is obtained and then the corresponding curve is measured, whereby, as a rule, a steep increase takes place in a relatively short time and the curve then flattens out as soon as the folding intermediate is formed. A further possibility consists in monitoring the disappearance of the SH groups by the formation of intramolecular disulphide bridges. However, it is hereby to be observed that no intramolecular disulphide bridges are to be formed, which can take place by monitoring the molecular weight which, in the

latter case, would increase.

A further possibility consists in the fluorescence measurement. This is based upon the tryptophane or tyrosine fluorescence which changes when the aromatic side-chains are transferred to a hydrophobic region of the folded protein. Further possibilities are limited by proteolysis since, with increasing formation of the folding intermediate, the proteolytic attack is also reduced. It is also possible to carry out this determination by hydrodynamic measurements. For example, there is here measured the intrinsic viscosity which decreases since the folding intermediate leads to the formation of a solution with lower viscosity.

In practice, it is sufficient to determine the critical concentration and the time up to the formation of the folding intermediate protected against the side reaction to inactive protein and, in each case, to commence with the critical concentration and, after termination of the formation of the folding intermediate, again to replenish the denatured protein consumed in the meantime from the solution by conversion. This can take place stepwise in that one predetermines the times and critical concentrations and then, in each case, after termination of the formation of the intermediate, there is replaced the known amount of denatured protein hereby changed. Alternatively, the process can also be carried out continuously in that there is newly added precisely just as much denatured protein as has disappeared by new formation of the folding intermediate. In this way, the renaturation can be carried out in a cyclic process in which, for example, one introduces the protein to be renatured continuously to the renaturation reactor dissolved in a solvent suitable therefor, e.g. highly diluted hydrochloric acid, or in lyophilised form, in each

case in such an amount that the optimum renaturation concentration is obtained. The renaturation buffer is simultaneously added continuously in such a composition that the conditions remain constant with regard to pH value, substance concentrations and the like. By appropriate construction of the reactor, which can be, for example, in the form of a loop, and the flow rate defined by the rate of addition and amount of protein and buffer, there can be defined a residence period which corresponds to the time up to the formation of the folded intermediate. Thereafter, reactivated protein is continuously removed, for example by separation by means of ultrafiltration, reverse osmosis and the like, and, as retentate, optionally subjected to a separation into inactive aggregate and active protein. The continuous process is preferably so carried out that the buffer solution separated off is again returned continuously to the reactor.

In the case of the continuous version of the process according to the invention, it is preferred initially, as in the case of the non-continuous process, to add the denatured protein in the above-described way until an optimum concentration of renatured active protein has been obtained. As soon as this is achieved, the continuous removal of the relatively concentrated solution of reactivated protein is commenced and only then one passes over into the actual continuous process. The initial phase, in which the concentration of reactivated protein still increases, is, therefore, introduced before the actual continuous process. As optimum concentration of renatured protein, there is thereby to be understood the highest possible concentration at which still no problems arise with insolubilisation of components of the reaction system.

In the process according to the invention, it is preferred to use lyophilised denatured protein. If, however, it is a question of a protein which only goes into solution slowly and with difficulty, then the
5 addition takes place in dissolved form in order definitely to be able to maintain the time characteristics of the process according to the invention.

By means of the process according to the invention, the undesired aggregate formation which
10 competes with the renaturation is admittedly minimised but not fully suppressed. However, the inactive aggregates formed have a substantially greater molecular weight and can, therefore, easily be removed from renatured protein by separation methods which utilise
15 this molecular weight difference, thus for example by molecular sieve fractionation, possibly ultrafiltration with suitable exclusion limits, gradient centrifugation and the like. Aggregates thus separated can then, after their solubilisation, again be introduced into
20 the renaturing process.

As renaturing buffer, there come into consideration those compositions known herefor. The pH value must lie within the range of the pH value at which the protein in question displays good stability, expediently
25 lies near the pH value of the maximum stability. In order to exclude the denaturing influence of impurities, for example of heavy metals, it is expedient to work in the presence of complex formers, such as EDTA and similar compounds. Thus, for example, in the
30 case of lactate dehydrogenase, there is usable phosphate buffer of pH 6 to 8 which contains about 1 mM EDTA and 1 mM DTE. For lysozyme, weakly alkaline tris/HCl buffer has proved useful which, besides EDTA, contains an SH group-containing substance and a small amount of
35 the corresponding disulphide. For the renaturing of

tPA, there is suitable, for example, the reactivation buffer described in German Patent Application P 3 537 708. For numerous other proteins, suitable renaturing buffers are known or can be found by the expert on the basis of the known properties of the protein, especially with regard to pH stability, content of SH groups, heavy metal sensitivity and the like, using a few preliminary experiments.

It was known that, for renaturation, certain limiting values should not be exceeded. New is the recognition that comparatively large amounts of denatured protein do not perhaps require larger amounts of solution volume in order to achieve larger amounts of renatured protein but that, in the same solution, by continuous new addition or intermittent new addition of denatured protein, one can work further and thus obtain substantially higher concentrations of renatured protein than was held to be possible according to the prior art.

The following Examples further explain the invention.

Example 1.

Pulse reactivation of lysozyme.

a) Preparation of the enzyme solutions.

Concentration determination (A: absorption of a 1% solution at 280 nm):

native lysozyme A = 26.3

reduced lysozyme A = 23.7

Determination of the specific activity:

Dissolve 2 mg lysozyme in 1 ml 0.066 mol/l K phosphate, pH 6.2; determine concentration by UV absorption.

Test solution: suspend 2.5 mg cell walls of *Micrococcus lysodeikticus* in 10 ml 0.066 mol/l K phosphate, pH 6.2.

Test at 25°C: 1 ml cell wall suspension plus 0.03 ml lysozyme solution (pre-diluted 1:40); monitor decrease of the turbidity at 450 nm.

Spec. activity = 60,000 IU/mg.

Reduction/denaturation:

Incubate 500 mg lysozyme for 3 h at 25°C in 0.1 mol/l tris/HCl, pH 8.6 + 6 mol/l Gdn hydrochloride + 150 mmol/l DTE.

Adjust pH to 3 and divide up sample.

Part A

Gel filtration over Sephadex G 25 F (column volume = 270 ml) in 0.01 mol/l HCl. Combine peak fractions and lyophilise.

Part B

Dialyse against 3 x 1 l 0.01 mol/l HCl at 4°C. Lyophilisation.

Both processes of desalination (part A and B) give reduced lysozyme which, with regard to reactivation properties, does not differ.

The lyophilised, reduced enzyme is stored at -40°C under N₂.

Reoxidation/reactivation:

Dissolve 12 mg reduced lysozyme in 1 ml 0.01 mol/l HCl.

Millipore filtration

Concentration determination by UV absorption.

Reoxidation at 25°C by dilution in 0.1 mol/l tris/HCl, pH 8.2 + 1 mmol/l EDTA + 3 mmol/l GSH + 0.3 mmol/l GSSG.

b) Determination of the critical concentration of the renaturation.

Reduction/reoxidation takes place as described above by variation of the protein concentration in the reactivation batch. Fig. 1 shows the result, where the concentration dependence of the reactivation of reduced lysozyme after 20 h of reoxidation at 25°C is illustrated graphically.

c) Time up to the formation of the folding intermediate.

By reduction/reoxidation as described above, there is determined the kinetics of the reactivation of reduced lysozyme at a protein concentration of 0.03 mg/ml; determined at 25°C. The result is illustrated graphically in Fig. 2.

d) "Pulse reactivation" of reduced lysozyme.

1.6 or 2.4 ml 0.125 mol/l tris/HCl, pH 8.2 + 1.25 mmol/l EDTA + 3.75 mmol/l GSH + 0.375 mmol/l GSSG are taken.

1st Experiment

In time intervals of $t = 10$ min, 20 x in each case 20 μ l of reduced lysozyme in 0.01 mol/l HCl are added. Concentration increase per pulse: 0.11 - 0.13 mg/ml. End concentration after 20 pulses: 2.15 mg/ml.

Comparative reactivation without pulse: single addition to the same end concentration. The buffer conditions correspond, after dilution, to those of the above-mentioned reoxidation buffer.

After 2 h reactivation at 25°C

pulse reactivation	2200 IU/mg
comparison without pulse:	470 IU/mg

2nd Experiment

In time intervals of $t = 10$ min, 20 x in each case 30 μ l of reduced lysozyme in 0.01 mol/l HCl are added. Concentration increase per pulse: 0.034 - 0.042 mg/ml. End concentration after 20 pulses: 0.68 mg/ml.

Comparative activation: single addition to the same end concentration.

After 2 h reactivation at 25°C:

pulse reactivation	7660 IU/mg
comparison without pulse	1400 IU/mg

3rd Experiment

In time intervals of $t = 20$ min, 20 x in each case 20 μ l of reduced lysozyme in 0.01 mol/l HCl are added. Concentration increase per pulse: 0.035 -

- 5 0.044 mg/ml. End concentration after 20 pulses: 0.71 mg/ml.

Comparative reactivation: single addition to the same end concentration

After 24 hours reactivation at 25°C:

- | | | |
|----|--------------------------|-------------|
| 10 | pulse reactivation | 10100 IU/mg |
| | comparison without pulse | 1400 IU/mg |

From these results, it follows that:

The reactivation yields in the case of protein concentrations of > 0.1 mg/ml decrease continuously.

- 15 At 25°C, the reactivation under the given reoxidation conditions is almost complete after 15 min (Fig.2).
By "pulse reactivation" according to the invention, in comparison with unpulsed reactivation, a 5 to 7 times higher yield can be achieved.

20 Example 2.

a) Preparation of the enzyme solution:

- LDH-M₄ (from pig muscle) suspension in $(\text{NH}_4)_2\text{SO}_4$ (producer: Boehringer Mannheim GmbH, Order No.107077) is centrifuged and the pellet taken up with 1/6 of the
25 original volume in 0.1 mol/l K phosphate, pH 7.6 + 1 mmol/l EDTA + 1 mmol/l DTE and dialysed against 2 x 1 l of the same buffer at 4°C. Concentration determination via $A = 14$ (A = absorption of a 1% solution at 280 nm).

- 30 The activity test at 25°C according to R. Herman, R. Jaenicke & R. Rudolph, Biochemistry, 20 (1981) 5195-5201, gives spec. activity = 300 IU/mg.

Denaturation/reactivation:

- Denaturation: 1:5 dilution with 0.1 mol/l H_3PO_4 ,
35 6 min incubation at 0°C.

Reactivation: 1:3 dilution with 0.1 mol/l K phosphate, pH 12.65 + 1 mmol/l EDTA + 1 mmol/l DTE at 20°C; after the mixing, the pH of the solution was 7.6.

5 b) Determination of the critical concentration of the renaturation.

Denaturation/reactivation take place as described above with variation of the protein concentration in the denaturing batch.

10 Fig. 3 shows the concentration dependency of the reactivation of LDH-M₄ after acid denaturation, whereby the activity after 20 h reactivation at 25°C is plotted against the concentration.

c) Determination of the time up to the formation of the folding intermediate.

15 By denaturation/renaturation as above, the kinetics of the reactivation of acid-denatured LDH-M₄ at a protein concentration of 0.01 mg/ml is determined at 25°C. Fig. 4 shows the result.

20 d) Continuous reactivation of acid-denatured LDH-M₄

Into a closed reactor, there was continuously fed in acid-denatured LDH-M₄ and reactivation buffer.

The reactivation reactor used is schematically illustrated in Fig. 5.

25 From storage vessels A and B, there is continuously fed in the solution of the enzyme and denaturing solution via pumps P₁ and P₂ and denatured in a mixing section V1. The denatured enzyme solution is continuously introduced into the renaturation reactor V2. At the same time, renaturation buffer from the
30 storage vessel C is also continuously fed via pump P₃ into the reactor V2. Renatured solution is continuously withdrawn and collected in the fraction collector 6. The composition of the solutions and the conditions present in the reactor were as follows:

- A: 7.43 mg/ml LDH-M₄ in 0.1 mol/l K phosphate,
pH 7.6 + 1 mmol/l DTE + 1 mmol/l EDTA at 0°C.
- B: denaturation buffer: 0.1 mol/l H₃PO₄ at 0°C.
- 5 C: renaturation buffer: 0.1 mol/l K phosphate,
pH 12.6 + 1 mmol/l DTE + 1 mmol/l EDTA at 25°C
(pH after mixing with B: pH 7.6).
- V1: denaturation volume V₁ = 0.25 ml. Gives a
denaturation period of 3 min. at 0°C.
- V2: renaturation volume V₂ = 58 ml; 25°C.
- 10 Pump velocity: P₁ = 1 ml/h
P₂ = 4 ml/h
P₃ = 10 ml/h.

In the case of the given reactor size/pump rate,
for the average residence time of a molecule in the
15 reactor, there is given 2.9 hours.

In the case of the run, 2.5 ml samples are
collected and the end reactivation determined after
24 h at 25°C.

Fig. 6 shows the activity increase in the case
20 of charging the reactor.

After about 4 h, a constant reactivation yield
is achieved.

end concentration of the reactivation: 0.5 mg/ml
yield: continuous reactivation: 10.2 IU/mg
25 comparison without pulse: 1.9 IU/mg

The above results show that, according to the
invention, there is achieved an approximately 5.4 times
higher yield than in the case of direct reactivation
with the same end concentration.

30 Example 3.

Pulse reactivation of rec-t-PA.

a) Preparation of the "refractile bodies (RB)"

"RB" preparation by cell digestion and diverse centrifuging/washing steps took place analogously to the process of EP A1-0114506, Example 7. t-PA is contaminated in the "RB's" with foreign protein,
5 inactive and insoluble.

Solubilisation by reduction/denaturation:

"RB's" from 2.5 g of cells incubated for 3 h at 25°C in 10 ml 0.1 mol/l tris/HCl, pH 8.6 + 6 mol/l Gdn-HCl + 1 mmol/l EDTA + 150 mmol/l DTE.

10 Total protein in the "RB's" after reduction:
41.5 mg.

The pH is adjusted to 3 with HCl conc. and gel filtered over Sephadex G 25 F (column volume = 270 ml) in 0.01 mol/l HCl.

15 b) Determination of the concentration dependency of the reactivation.

The acidic fraction of the reduced protein with maximum recovery of activity ($c = 0.83$ mg/ml) is reoxidised by 1:3 to 1:500 dilution in 0.1 mol/l
20 tris/HCl, pH 10.4 + 1 mmol/l EDTA + 0.5 mol/l L-arginine + 1 mg/ml BSA + 1 mmol/l GSH + 0.2 mmol/l GSSG.

The reoxidation conditions in the case of various dilutions are constantly adjusted. After 25 - 27 h
25 reoxidation at 25°C, the activity is determined. Before the activity measurement, it is adjusted with reoxidation buffer to the same end concentration. Table 1 and Fig. 7 of the drawing show the results.

Table 1
Concentration dependency of the reactivation of
rec-t-PA

	protein concentration ¹ (ug/ml)	yield ²	stimulatability (factor)
5	277	31.3	16.8
	138	52.4	17.0
	92.2	64.9	18.5
10	55.3	74.9	21.5
	27.7	91.5	17.5
	17.3	95.6	25.3
	8.68	100.0	20.5
	5.53	97.8	19.8
15	3.95	94.4	20.6
	1.84	85.3	16.7

¹protein concentration in the case of reactivation
(total protein)

²referred to maximum reactivation value

- 20 c) Determination of the kinetics of the reactivation.
Reduction/reoxidation are carried out as above.

Because of the impurity of the material, the
time up to the formation of the folding intermediate
cannot be measured directly but rather indirectly via
25 the kinetics of the reactivation and variation of the
pulse rate to about 40 to 60 minutes.

Fig. 8 shows the kinetics of the reactivation
of rec-t-PA at a total protein concentration of 15 ug/ml
at 20°C. Reactivation referred to final yield after
25 30 h.

- d) "Pulse reactivation" of rec-t-PA.

In time intervals between 2 min and 45 min,
15 x in each case 10 µl of reduced rec-t-PA in

0.01 mol/l HCl are introduced into 300 µl reactivation buffer (25°C).

Reactivation buffer before pulse reactivation:

0.15 mol/l tris/HCl, pH 10.5 + 1.5 mmol/l EDTA +

5 0.5 mol/l L-arginine + 0.3 mmol/l GSSG + 1.5 mmol/l GSH.

Reactivation buffer after addition of the reduced

protein: 0.1 mol/l tris/HCl, pH 10.4 + 1 mmol/l EDTA +

0.33 mol/l L-arginine + 0.2 mmol/l GSSG + 1. mmol/l GSH.

10 Concentration increase per pulse: 18 to 27 µg/ml

End concentration after 15 pulses: 227 µg/ml

Activity measurement takes place after 24 h reoxidation at 20°C. Table 2 shows the results.

Table 2

15 Pulse reactivation of rec-t-PA

	pulse rate (min)	yield ¹	stimulatability (factor)
	2	31.3	7.1
	10	38.6	8.1
20	20	39.5	9.0
	30	44.8	8.2
	45	45.8	7.8

¹Referred to maximum reactivation corresponding to Fig.7.

25 Commentary:

In the case of the starting material used, the reactivation yield - referred to the amount of protein used - decreases at concentrations of >20 µg/ml

(Figure 7). The stimulatability by CNBr-FSP proves,
30 in the range of measurement exactitude, to be independent of the reactivation concentration (Table 1).

At 20°C, the half life value of the reactivation is, under the given experimental conditions, 7 ± 2 hours.

The reactivation yield increases with increasing residence time between the reactivation pulses (Table 2).



Patent Claims

1. Process for the renaturation of denatured proteins in solution in a renaturation buffer, characterised in that one prepares a solution of the protein to be renatured in the concentration in which, in the selected buffer, the highest yield of native renatured protein is obtained (critical concentration) and, after formation of the folding intermediate, adds further protein to be renatured in the amount necessary for the achievement.
2. Process according to claim 1, characterised in that the process is carried out discontinuously in that, in each case after expiry of the time necessary for the formation of the folding intermediate, the amount of denatured protein necessary for the achievement of the critical concentration is added thereto.
3. Process according to claim 1, characterised in that, for the continuous renaturation, into a reactor are continuously introduced the amount of denatured protein for the achievement of the critical concentration and the corresponding amount of renaturation buffer and the residence time of the mixture in the reactor is adjusted to the period needed for the formation of the folding intermediate.
4. Process according to claim 3, characterised in that the denatured protein is left in the reactor until the highest possible concentration of renatured protein is reached at which still no components of the reaction system become insoluble (optimum concentration) and then the so obtained solution is removed continuously and correspondingly replaced by a solution of denatured protein in optimum concentration.
5. Process according to one of the preceding claims, characterised in that inactive protein aggregate formed is separated off on the basis of its higher molecular



-19-

weight from renatured protein and, after dissolving, again introduced into the process.

6. Process according to one of the preceding claims, characterised in that the denatured protein
- 5 is introduced into the process in lyophilised or pre-dissolved form..