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RENATURATION AND UREA-INDUCED DENATURATION OF 20β-HYDROXYSTEROID DEHYDROGENASE STUDIED IN SOLUTION AND IN THE IMMOBILIZED STATE

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Summary

Tetrameric 20β-hydroxysteroid dehydrogenase (17,20β,21-trihydroxysteroid:NAD^+ oxidoreductase, EC 1.1.1.53) from *Streptomyces hydrogenans* was reactivated after inactivation, dissociation and denaturation with urea. The effect of several factors such as NAD^+, NADH, substrate, sulphydryl reducing agents, extraneous proteins, pH and enzyme concentration on reactivation was investigated. The coenzymes were found to be essential for obtaining a high reactivation yield (about 90%), since in their absence the reactivation was less than 10%. NADH was effective at lower concentrations than NAD^+. The reactivated enzyme, after the removal of inactive aggregates, showed physical and catalytic properties coincident with those of the native enzyme.

The mechanism by which NADH affects the reconstitution of 20β-hydroxysteroid dehydrogenase was investigated using both soluble enzyme and enzyme immobilized on Sepharose 4B. The immobilization demonstrates that isolated subunits are inactive and incapable of binding NADH and suggests that subunit association to the tetramer is essential for enzymatic activity. NADH appears to act, after subunit assembly has taken place, by stabilizing tetramers and preventing their aggregation with monomers that would give rise to inactive polymers.

Introduction

20β-hydroxysteroid dehydrogenase (17,20β,21-trihydroxysteroid:NAD^+ oxidoreductase, EC 1.1.1.53) from *Streptomyces hydrogenans* is a NAD-depen-

dent enzyme [1,2], consisting of four apparently identical subunits [3].

The enzyme appears to be a good model for the study of reactivation and subunit assembly of oligomeric proteins, particularly of NAD-dependent enzymes, after inactivation and dissociation with denaturing agents. In the present work the effect of several factors such as coenzymes, substrate, sulphydryl reducing agents, extraneous proteins, pH and enzyme concentration on the reactivation of 20β-hydroxysteroid dehydrogenase after denaturation by 6 M urea has been investigated. As previously suggested for various other proteins [4—11] these factors could affect the folding and association of subunits in vitro and perhaps in vivo.

The effect of coenzymes on 20β-hydroxysteroid dehydrogenase has been examined with particular care, because NAD^+ was found to markedly increase the reactivation of glyceraldehyde-3-phosphate dehydrogenase [6,7,10], while the same effect on lactic dehydrogenase and malate dehydrogenase was reported by some authors [6] and denied by others [8,12].

In this work the enzyme has been studied under two different conditions, in solution and immobilized on CNBr-activated Sepharose 4B.

The immobilization was performed in such a way that, preferentially, a single subunit per tetramer would be linked covalently to the matrix. Non-covalently linked subunits were removed with urea and then the immobilized subunits were renatured under suitable conditions. This method, first used by Chan with aldolase [13] and subsequently by several other authors with various enzymes (for a review see Ref. 24), is very useful because it makes it possible to examine, under nondissociating conditions, properties, such as activity and binding capacity for ligands, of isolated subunits.

Materials

NAD^+, NADH and 20β-hydroxysteroid dehydrogenase with specific activities of about 10 U/mg were obtained from Boehringer. The enzyme was homogeneous on disc-gel-electrophoresis and on gel-filtration. Before use the enzyme was exhaustively dialysed at 4°C against 0.05 M potassium phosphate buffer, pH 7, containing 1 mM dithiothreitol. Urea (for biochemistry) was obtained from Merck and Sepharose 4B from Pharmacia. 6β-Bromoacetoxyprogesterone was synthesised according to Arias et al. [14]. All other reagents and compounds were analytical grade.

Methods

Analyses. The concentration of soluble enzyme was determined by the method of Lowry et al. or calculated from $E^{1\%}_{280} = 9.33\ cm^{-1}$. The concentration of immobilized enzyme was determined by amino acid analysis after hydrolysis of measured samples with 6 M HCl, for 24 h at 110°C [15,16]. The volume of the gel was measured after centrifugation for 3 min at 1500 × *g*. The activity of the soluble enzyme was determined spectrophotometrically in 0.05 M potassium phosphate buffer pH 7/0.12 mM NADH/0.1 mM cortisone. The activity of the immobilized enzyme was determined as above using continuous stirring [17] and higher concentrations of NADH (0.17 mM) and cortisone (0.18 mM).

Fluorescence was measured in a Jasco FP550 spectrofluorimeter. Circular dichroism spectra were recorded over a wavelength range of 220—300 nm with a Cary 61 spectropolarimeter.

Affinity labelling of 20β-hydroxysteroid dehydrogenase with 6β-bromoacetoxyprogesterone. 6 mg aliquots of enzyme in 10 ml of 0.05 M potassium phosphate buffer, pH 7, were reacted with 540 μg of 6β-bromoacetoxyprogesterone in 0.6 ml ethanol, at 25°C [14]. When complete inactivation was obtained (after about 5 h) the enzyme was dialysed overnight in the cold against distilled water and concentrated under vacuum at room temperature. The activity of the treated enzyme was found to be completely absent. Controls were made by treating the enzyme as above but replacing 6β-bromoacetoxyprogesterone with progesterone. Activity recovery was 60—70%.

Inactivation and reactivation. Enzyme inactivation was obtained by incubation at 25°C for 1 h, with 6 M urea in standard buffer (0.05 M potassium phosphate buffer, pH 7/1 mM dithiothreitol) unless otherwise stated. The inactivated enzyme was reactivated at 25°C by a ⩾60-fold dilution with 0.05 M potassium phosphate buffer, pH 7, in the presence or absence of additives. Aliquots were taken at defined times and assayed for activity. The activity was expressed as a percentage of the activity of the native enzyme under comparable conditions.

Immobilization of 20β-hydroxysteroid dehydrogenase. CNBr-activated Sepharose 4B was prepared according to Axen et al. [18] but using lower CNBr concentrations (3—10 mg CNBr/ml of settle gel). The coupling reaction was carried out overnight, with gentle stirring, in 0.1 M potassium phosphate buffer, pH 8.5, at 4°C using 3 mg of 20β-hydroxysteroid dehydrogenase per ml of activated gel, in the presence of 0.15 mM NADH. The latter was found to increase the recovery of the immobilized activity [17]. Blockage of remaining reactive groups was achieved by treatment with 0.08 M ethanolamine, pH 8.5, for 4 h. The immobilized tetrameric enzyme was washed thoroughly with cold standard buffer and tested for enzymatic activity and protein content. A reference Sepharose was prepared in the same way but without the addition of the enzyme.

Preparation of immobilized subunits and their interaction with soluble subunits. The immobilized tetrameric enzyme was incubated at 25°C, for 1 h, with 6 M urea in standard buffer and then washed in a sintered-glass funnel with several volumes of the same buffer in order to remove subunits not covalently linked to the matrix. To promote renaturation, the gel was washed extensively with standard buffer in the presence or absence of 1 mM NADH and incubated overnight at 20°C. The immobilized subunits thus obtained were tested for enzymatic activity and protein content.

The immobilized subunits, suspended in a suitable volume of standard buffer plus 1 mM NADH, were gently mixed overnight, at 20°C, with an 8—10-fold excess (on protein weight basis) of soluble subunits, obtained by treatment with 6 M urea of the tetramer labelled or not with 6β-bromoacetoxyprogesterone. The soluble subunits were added in three portions at 30 min intervals. The final urea concentration was less than 0.1 M. The reassociated derivative was then washed extensively with standard buffer and tested for enzymatic activity and protein content. The reference Sepharose was processed in the

same way. The principles underlining the preparation of the derivatives described above have been illustrated in detail by Chan [19].

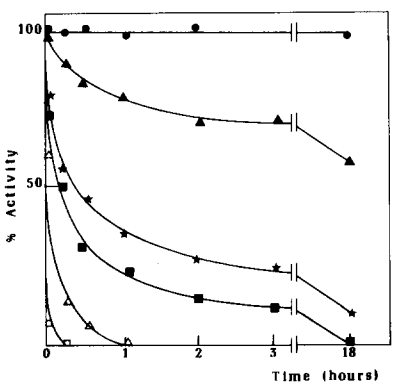
Measurement of NADH binding to immobilized tetrameric enzyme and to immobilized subunits. Following the method described by Swaisgood et al. [20] aliquots of immobilized tetrameric enzyme and of immobilized subunits (500—1000 μg protein) were equilibrated, at 20°C, with NADH solutions of various concentrations. The concentration of free NADH was then determined by spectrophotometric measure of the supernatant at 250 nm. Bound NADH was inferred by subtracting free NADH from total NADH added.

Results

Inactivation of 20β-hydroxysteroid dehydrogenase

Fig. 1 shows the time course of inactivation of the enzyme as a function of urea concentration. Rapid inactivation is achieved at urea concentration ⩾2 M; the other concentrations tested, except 0.15 M urea, which does not affect activity, give rise to slow but progressive inactivation.

NADH was found to markedly protect the enzyme against inactivation by urea approaching its maximum effect at 1.5—2 mM concentration (Fig. 2). The insert to Fig. 2 shows that in the presence of 1.5 mM NADH the urea concentration necessary to reduce enzyme activity to 50% is about 3-times higher than that needed in the absence of cofactor. NAD^+ produced a similar protecting effect but at concentrations 3—4-times higher than NADH. The increase of enzyme concentration also increased the concentration of urea necessary for the inactivation (data not shown). However, using 6 M urea the complete



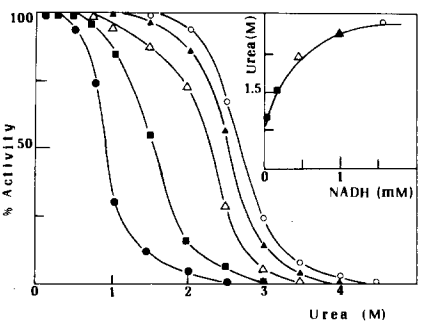


Fig. 1. Time course of inactivation of 20β-hydroxysteroid dehydrogenase by urea. The enzyme (10 μg/ml) was incubated at 25°C in 0.05 M potassium phosphate buffer, pH 7/1 mM dithiothreitol, containing 0.15 M (●———●), 0.5 M (▲———▲), 0.8 M (★———★), 1 M (■———■), 2 M (△———△) and 4 M (□———□) urea. Enzyme activity was measured in the presence of urea at the same concentration used for incubation and expressed as percentage of the activity of the enzyme incubated in the absence of urea.

Fig. 2. Inactivation of 20β-hydroxysteroid dehydrogenase by urea as a function of NADH concentration. The enzyme (14 μg/ml) was incubated at 25°C for 1 h in 0.05 M potassium phosphate buffer, pH 7, 1 mM dithiothreitol, containing the scheduled concentrations of urea and NADH. Enzyme activity was measured as reported in the legend to Fig. 1. No NADH present (●———●), 0.1 mM NADH (■———■), 0.5 mM NADH (△———△), 1 mM NADH (▲———▲) and 1.5 mM NADH (○———○).

enzyme inactivation was always achieved within a few minutes.

The enzyme inactivated by 6 M urea, filtered on a Sephadex G-200 column (1 × 95 cm) equilibrated with the standard buffer plus 6 M urea, showed a single peak. The peak, as inferred from a calibration curve obtained by gel-filtration of proteins of known M_r, had an apparent M_r of 28 000 which corresponds to the reported subunit M_r [3].

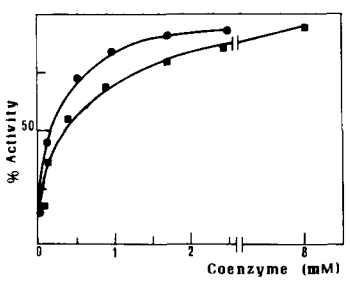
The fluorescence spectra of native and inactivated enzyme, obtained by excitation at 280 nm, were markedly different, with an emission maximum at 330 and 350 nm, respectively. The relative intensity of emission of the inactivated enzyme was 54% that of the native one, at 330 nm. Circular dichroism spectra showed a very marked decrease of the α-helical content of the inactivated compared with the native enzyme. Thus gel filtration, fluorescence and CD experiments indicate that inactivation is associated with dissociation and unfolding.

Reactivation of 20β-hydroxysteroid dehydrogenase

The reactivation yield of 20β-hydroxysteroid dehydrogenase in standard buffer alone was found to be less than 10%. The presence of NAD^+ and NADH highly increased the reactivation. Fig. 3 shows the dependence of the reactivation yield on the concentration of coenzymes. This measurement was made after 20 h reactivation time at which the process was practically finished.

NADH was more effective at lower concentrations than NAD^+ (Fig. 3) and so it was chosen as the coenzyme to be used for subsequent studies.

Fig. 4 shows the time course of reactivation of the enzyme with NADH added at different times. Maximum effect was obtained by adding the coenzyme at the beginning of the process. When the addition was made after 150 min less effect was noted and when the addition was made after 14 h there was practically no effect. This demonstrates that irreversible processes



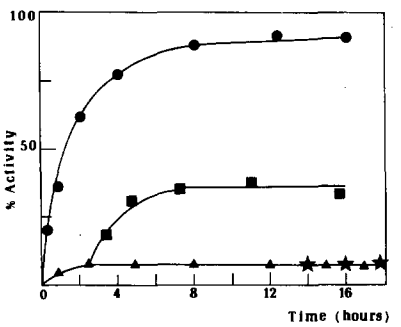
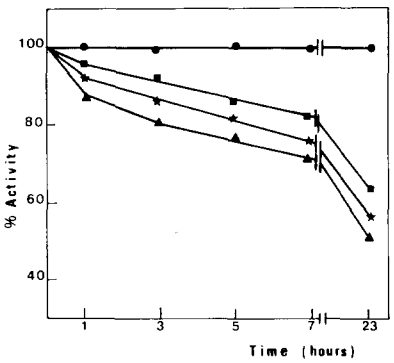


Fig. 3. Dependence of the reactivation of 20β-hydroxysteroid dehydrogenase on coenzyme concentration. Reactivation of the urea inactivated enzyme was initiated by a 60-fold dilution with the standard buffer containing the indicated NADH (●——●) or NAD^+ (■——■) concentration. The enzyme concentration was 14 μg/ml and the reactivation time 20 h.

Fig. 4. Dependence of the reactivation of 20β-hydroxysteroid dehydrogenase on the time of NADH addition. Enzyme reactivation was initiated by a 80-fold dilution with the standard buffer in the presence (●——●) or absence (▲——▲) of 1 mM NADH. The same NADH concentration was added to the medium initially devoid of coenzyme 150 min (■——■) and 14 h (★——★) after the start of reactivation. The enzyme concentration was 12 μg/ml.



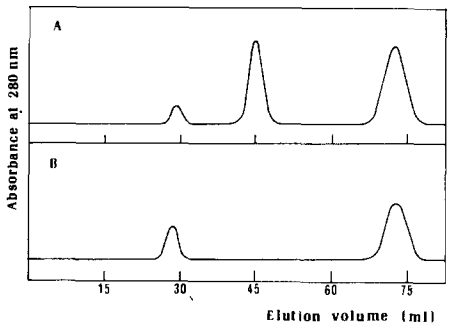


Fig. 5. Effect of the urea denatured enzyme on the activity of native 20β-hydroxysteroid dehydrogenase. 20β-hydroxysteroid dehydrogenase completely inhibited by affinity labelling with 6β-bromoacetoxyprogesterone was treated with 6 M urea in standard buffer for 1 h at 25° C and then diluted (60-fold) with standard buffer containing native 20β-hydroxysteroid dehydrogenase in the presence (●) or absence (■, ★, ▲) of 1 mM NADH. The concentration of native enzyme was 15 μg/ml in all cases. The concentration of affinity labelled enzyme was 15 (■), 30 (●, ★) and 45 (▲) μg/ml. Activity was measured at the indicated times and expressed as percentage of the activity of controls made under the same conditions but with no addition of denatured enzyme.

Fig. 6. Gel filtration profile of 20β-hydroxysteroid dehydrogenase reactivated in the presence (A) and absence (B) of NADH. Reactivated enzyme was gel filtered on a column (1 × 100 cm) of Bio-Gel A-1.5 m, equilibrated with 0.05 M potassium phosphate buffer, pH 7. The flow rate was 3.5 ml/h. The enzyme reactivated in the presence of NADH (1 mM), and used for the gel filtration experiment, gave a 88% reactivation yield while the enzyme reactivated in the absence of NADH gave a 6% reactivation yield.

take place progressively in the absence of the cofactor.

It was also found that in the absence of NADH the native enzyme was inactivated by the urea-denatured enzyme added under reconstituting conditions (Fig. 5). The inactivation increased as a function of time and of concentration of denatured enzyme. It should be noted that no activity contribution from the denatured enzyme was possible during the process because the enzyme used was completely inhibited by the affinity labelling.

The characteristics of the enzyme reactivated in the presence of NADH were examined after dialysis, concentration under vacuum and gel filtration. The gel filtration gave a pattern of three peaks (Fig. 6A). The first, eluted in the void volume, was inactive, the second, which was active and had an elution volume, i.e., a molecular weight, identical to that of the native enzyme, was considered the reactivated enzyme, and the third which was composed of NADH and dithiothreitol was not completely removed by dialysis. The specific activity of the reactivated enzyme was 97% that of the native one. The fluorescence spectra and K_m values for cortisone and NADH of the reactivated and native enzyme were the same, within experimental error. All these data indicate that the reactivated enzyme reacquires the native quaternary structure.

The gel filtration pattern of the enzyme reactivated in the absence of NADH showed that there was no detectable material with an elution volume coincident with that of the native enzyme (Fig. 6B). It should be noticed that insoluble material was observed in the reactivation mixture after dialysis and concentration.

The effect of other factors on enzyme reactivation was investigated. Bovine

serum albumin (from 0.1 to 5 mg/ml), cortisone (from 0.1 to 1 mM) and pH (from 6.5 to 9) did not influence or scarcely influenced the reactivation process. Dithiothreitol (from 1 to 15 mM) slightly stabilized the enzyme but did not affect the reactivation appreciably. The enzyme concentration, by contrast, markedly influenced the reactivation rate. This suggests that an association reaction is involved in the reactivation of 20β-hydroxysteroid dehydrogenase.

Properties of immobilized tetrameric enzyme and of immobilized subunits

The specific activity of immobilized 20β-hydroxysteroid dehydrogenase was about 75% that of the soluble enzyme. Apparent K_m values for cortisone and NADH of the immobilized enzyme were, as previously reported [17], considerably higher than those of the soluble enzyme. This suggests that the activity of the immobilized enzyme is restricted, at least in part, by diffusion limitations.

The effect of the degree of activation on the protein content and on the relative enzymatic activity of the immobilized derivatives: tetrameric enzyme, subunits and reassociated enzyme, is illustrated in Table I. The protein content of immobilized subunits was 35% that of the tetrameric enzyme in the lower activation experiment and 54% in the higher activation experiment. This indicates that in both cases a more or less large portion of the enzyme was covalently linked to the matrix through two or more subunits. The immobilized subunits did not show any significant activity whether they were renatured in the presence of NADH or not (the activity was less than 0.5% that of the tetrameric enzyme). The addition of soluble subunits, completely inhibited with 6β-bromoacetoxyprogesterone, restored 22 or 32% of the original activity depending on the degree of activation. These values, which correspond respectively to 63 and 59% of the values theoretically achievable with a complete reactivation of the subunits covalently linked to the matrix, are in good agreement with the reactivation yield obtained by adding soluble subunits not inhibited by affinity labelling (Table I).

The reactivation due to the addition of soluble subunits was found to be identical whether the renaturation process of immobilized subunits was performed in the absence of NADH or not.

Reactivation yield and protein recovery of immobilized subunits following reassociation with soluble subunits were strictly connected (Table I).

TABLE I

PROTEIN CONTENT AND RELATIVE ENZYMATIC ACTIVITY OF IMMOBILIZED DERIVATIVES

The values obtained using uninhibited subunits are in parentheses.

mg CNBr/ ml Sepharose	Tetrameric enzyme		Subunits		Enzyme reassociated with inhibited subunits	
	μg protein/ ml Sepharose	% activity	μg protein/ ml Sepharose	% activity	μg protein/ ml Sepharose	% activity
3	210	100	74	0	150 (145)	22 (60)
10	576	100	314	0	458	32

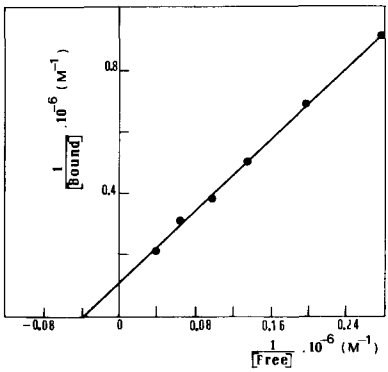


Fig. 7. Double-reciprocal plot of NADH binding to the immobilized tetrameric enzyme. Aliquots of immobilized 20β-hydroxysteroid dehydrogenase containing 1.07 mg of protein were equilibrated with various NADH concentrations in 4 ml of 0.05 M potassium phosphate buffer, pH 7. Binding of NADH was then determined as described in the Methods section.

The lower reactivation yield obtained with the immobilized enzyme (approx. 60%) compared with that of the soluble one (90%, see Fig. 4), would be due, among other reasons [21,22], to a faster and preferential association of soluble added subunits with themselves rather than with immobilized ones.

Derivatives were also prepared using activations lower than 3 mg CNBr/ml Sepharose. These derivatives however gave no reliable results because the amounts of enzyme immobilized on the matrix were too small.

As expected reference Sepharose did not show any activity with the addition of soluble subunits.

The above experiments, particularly those making use of subunits labelled with 6β-bromoacetoxyprogesterone, clearly show that isolated subunits are intrinsically inactive and that subunit association is essential for enzymatic activity. The fact that the immobilized subunit derivative containing 54% of the original protein, which corresponds to an average of covalently linked dimers, is inactive (Table I), suggests that the activity is displayed only by oligomers higher than dimers, namely by tetramers.

The measurement of NADH binding to the immobilized tetrameric enzyme gave a dissociation constant value of 26 μM (Fig. 7) which is in fairly good agreement with the value of 16 μM found with the soluble enzyme [23]. This indicates that the immobilization process does not markedly alter the affinity of NADH for the enzyme.

1 mol tetrameric enzyme (on a molecular weight basis of 110 000) binds, at saturation, 3.6 mol NADH (Fig. 7). The discrepancy with the theoretical value of 4 mol, could be due to experimental errors or to the fact that a small fraction of the immobilized enzyme is inactive and incapable of binding NADH.

Immobilized subunits, by contrast, did not show any detectable binding capacity for NADH.

Discussion

The results reported in this paper contribute to the knowledge of the denaturation and renaturation processes of 20β-hydroxysteroid dehydrogenase and

of NAD-dependent dehydrogenases in general. In concentrated urea solutions denaturation and inactivation are associated with dissociation into subunits and reassembly to the tetramer appears to be a key step in the renaturation process.

The results demonstrate that 20β-hydroxysteroid dehydrogenase can be fully reconstituted after inactivation, dissociation and unfolding by urea. In fact the physical and catalytic properties of the reactivated enzyme, after the removal of inactive polymers, are coincident with those of the native enzyme.

Contrary to the other parameters examined, which were found to have little or no effect, the cofactor (NAD^+ or NADH) is required in order to obtain a high reactivation yield in vitro.

Several hypotheses may be considered to explain the mechanism of action of the cofactor. There could be a binding of the cofactor to a nucleation site of the refolding subunit (first hypothesis). The binding would stabilize the nucleation site thus accelerating the kinetics of refolding or would prevent an irreversible nonspecific folding leading to an incorrect monomer which subsequently polymerizes. This hypothesis is maintained by some authors [5—7] to explain the effect of NAD^+ on lactic dehydrogenase, malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase reactivation. A second possibility is that the coenzyme promotes subunit assembly by binding to correctly folded subunits (second hypothesis). A third hypothesis, suggested by Rudolph et al. [10] for glyceraldehyde-3-phosphate dehydrogenase, is that the coenzyme binds to the assembled tetrameric enzyme and prevents unfolded or refolded monomers aggregating with tetramers and giving rise to inactive polymers.

The first hypothesis is to be rejected for the following reasons, immobilized subunits reactivate identically with the addition of soluble subunits whether they have been renatured in the presence of NADH or not. This finding points to a correct refolding of isolated subunits in the absence of NADH. Moreover the inability of immobilized subunits to bind NADH makes the possibility that an early semistructured form of the subunit is able to bind the cofactor very improbable. This also disproves the second hypothesis.

On the contrary the validity of the hypothesis that NADH prevents the interaction of the reassembled tetramer with intermediates of the reconstitution pathway is also supported, besides the above considerations, by the finding that the native tetramer is inactivated by the reconstituting enzyme when NADH is omitted (Fig. 5).

In conclusion the coenzyme, following the binding to the enzyme, would stabilize and tighten the quaternary structure of 20β-hydroxysteroid dehydrogenase thus avoiding the formation of inactive polymers. Furthermore using simple thermodynamic considerations it may also be expected that, if the tetramer has a higher affinity for the coenzyme than the monomer, the presence of coenzyme would favour tetramer formation notwithstanding kinetic effects.

It is at present an open question whether NAD^+/NADH also have a role in the in vivo assembly of 20β-hydroxysteroid dehydrogenase.

The experiments performed using the immobilized enzyme aiming to clarify the mechanism of action of the coenzyme, also unequivocally demonstrate that the isolated subunits are inactive and incapable of binding NADH and suggest that the association to the tetramer is essential for the full expression of the catalytic properties.

On the basis of the results obtained and of the considerations just made, the processes involved in the denaturation and renaturation of 20β-hydroxysteroid dehydrogenase may be summarized in the following scheme:

Inactive denaturated monomer ⇌ inactive renatured monomer
Inactive renatured monomer ⇌ active tetramer
Active tetramer + NADH ⇌ NADH bound active tetramer
Inactivate denatured monomer → inactive aggregates
Inactive denatured monomer + active tetramer → inactive aggregates

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