KINETIC ANALYSIS OF THE REACTIVATION OF RABBIT MUSCLE ALDOLASE AFTER DENATURATION WITH GUANIDINE·HCL

Rainer RUDOLPH, Eric WESTHOF, and Rainer JAENICKE
Institut für Biophysik und Physikalische Biochemie, FBB, Universität Regensburg, Universitätsstraße 31, D-8400 Regensburg, FRG

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1. Introduction

Previous investigations [1,2] of rabbit muscle aldolase (fructose-1,6-biphosphate: D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) were concerned with the renaturation and reactivation after acid dissociation. A high yield of reactivation was obtained, the reactivated enzyme being indistinguishable from the native tetramer according to available biochemical and physico–chemical criteria [1]. Renaturation, as monitored by protein fluorescence, is a multi-step process composed of a fast concentration-independent increase in fluorescence emission and a slow concentration-dependent decrease which parallels the recovery of enzymic activity.

At high enzyme concentration the reactivation obeys first-order kinetics; at lower concentration a transition to a higher reaction-order is observed. This (in connection with the specific features of the reactivation kinetics) suggests an intermediate with partial activity while full activity and native fluorescence require association.

Earlier experiments by Chan et al. [3–5] pointed in the same direction. Kinetic analysis by Teipel [6] showed that first-order reactivation and reassociation parallel each other. This accords with Chan's experiments where reactivation was tested in the presence of trypsin or 2.3 M urea [4] indicating full enzymic activity of the monomer. This result contradicts conclusions drawn from our previous reactivation studies [2]. Therefore, we applied Chan's approach in a concentration range sufficiently wide to unequivocally determine the reaction-order and residual activity of the different states of association of the enzyme.

Both, the total reactivation and formation of the tetramer, tested in the presence of trypsin (A) or 2.3 M urea (B) were concentration-dependent.

The results may be described by an irreversible consecutive reaction $2\,D \rightarrow 2\,D^* \rightarrow N$, where the intermediate D* is calculated to show 50±10% specific activity.

2. Materials and methods

Fructose-1,6-biphosphate aldolase from rabbit muscle, fructose-1,6-biphosphate, NADH and the mixture of glycerol-3-phosphate dehydrogenase and triose-phosphate isomerase for the coupled assay were purchased from Boehringer (Mannheim), bovine serum albumin (BSA) and sucrose from Serva (Heidelberg), ultrapure guanidine·HCl and urea from Schwarz-Mann (NY). Trypsin (Boehringer) was treated with L-(1-tosylamido-2-phenyl) ethylchlormethyl ketone (Serva) [8]. All other reagents were of A-grade purity (Merck, Darmstadt).

Stock solutions of the enzyme (∿ 3 mg/ml) were prepared by repeated dialysis at 4°C against 0.1 M Tris–HCl buffer pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol. Enzyme concentration was calculated from $A_{280}^{1\%} = 9.1\ cm^2.mg^{-1}$ [9]. Aldolase activity was measured according to [10]. For reactivation kinetics the test was modified according to Chan et al. [7]. Test A contained 20 μg/ml trypsin, test B 2.3 M urea.

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The native enzyme had a spec. act. 10.7 IU/mg in test A and 9.9 IU/mg in test B, respectively. Enzyme concentration specific activity were determined with a Zeiss DMR 10 spectrophotometer thermostated at 25°C.

Incubation of the enzyme in 6 M guanidine·HCl + 1 mM EDTA + 10 mM dithiothreitol for 2 h at room temperature; reactivation by dilution in 0.05 M Tris–HCl buffer, pH 7.5 + 1 mM EDTA + 0.14 M mercaptoethanol + 1 mg/ml BSA + 20% sucrose at 0°C as described by Chan et al. [7].

3. Results and discussion

As in the case of the reactivation after acid dissociation [1] the reactivation after treatment with 6 M guanidine·HCl at enzyme concentration 1–50 μg/ml yields up to 85%. Since the enzyme in the presence of BSA remains stable over a long period of time (> 7 days) even at the lowest concentration, the final value of reactivation could be determined with high precision.

To follow the kinetics of reactivation samples were withdrawn at defined time intervals and subjected to the two tests introduced by Chan et al. [7]. This allows differentiation between maximum reactivation on one hand and activity due to tetramer formation on the other. In the trypsin test (A) folded polypeptide chains are stable regardless of their state of association. Only unfolded chains are cleaved and thus prevented from reactivation in the enzymic test; they do not contribute to the final activity which, therefore, represents the maximum yield of reactivation, i.e., the activity of tetramers and refolded smaller entities. The test in the presence of 2.3 M urea (B) is a quantitative measure for the amount of enzymically active tetramers present at a given time, since all dissociation products are unstable under the given solvent conditions [11].

3.1. *Tests in the presence of trypsin*

The kinetics of the reactivation of tetramers and refolded smaller entities show a significant enzyme concentration-dependence at 3–50 μg/ml (fig.1). Similar to the reactivation after acid dissociation [2] the reaction is biphasic. A concentration-independent rapid increase of specific activity is followed by a second phase which is significantly slowed down at low concentrations.

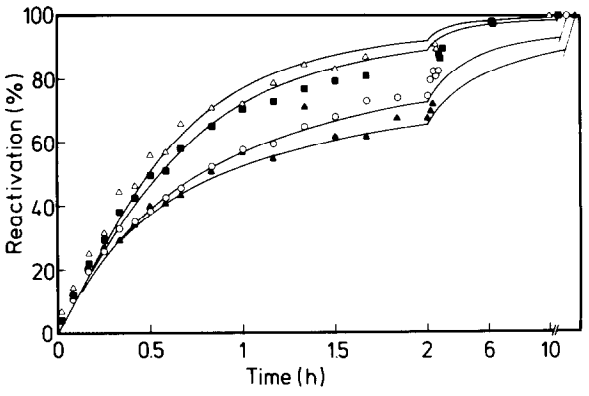

Fig.1. Kinetics of reactivation of rabbit muscle aldolase after deactivation in 6 M guanidine·HCl. Reactivation kinetics at varying enzyme concentrations (△) 50 μg/ml, (□) 25 μg/ml, (○) 6 μg/ml, (▲) 3 μg/ml. Activity tested in the presence of trypsin. Solid lines were calculated with $k_1 = 7.4.10^{-4}$ [sec^{-1}] and $k_2 = 1.4$ [$mM^{-1}.sec^{-1}$] and 50% residual activity for D*.

3.2. *Test in 2.3 M urea*

The formation of active tetramers shows a pronounced enzyme concentration-dependence and a sigmoidal profile of its time-course (fig.2).

3.3. *Kinetic model of the reactivation*

The reassociation of a tetrameric enzyme may

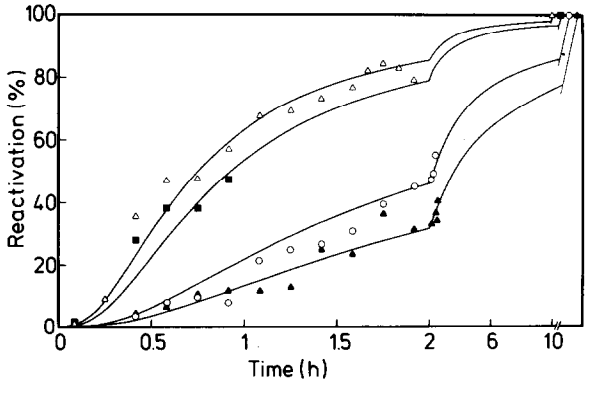

Fig.2. Kinetics of reactivation of rabbit muscle aldolase after deactivation in 6 M guanidine·HCl. Reactivation kinetics at varying enzyme concentrations (Symbols as in fig.1). Activity tested in the presence of 2.3 M urea. Solid lines were calculated with $k_1 = 7.4.10^{-4}$ [sec^{-1}] and $k_2 = 1.4$ [$mM^{-1}.sec^{-1}$].

include a sequence of transconformation and reassociation steps [12]. In the present case the differences in the kinetics of the reactivation (A) and the kinetics of tetramer formation (B) suggest at least partial activity of intermediary species. As shown by the concentration dependence of the reactivation of tetramers and refolded smaller entities, generation of full enzymic activity obviously includes an association reaction as rate-determining step; therefore, the intermediate cannot be fully active.

The simplest kinetic model to describe the given findings would include a sequence of two rate-determining irreversible reactions of first and second-order

$$2D \xrightarrow{k_1} 2D^* \xrightarrow{k_2} N \qquad (1)$$

with D, D* inactive and partially active denatured states and N fully active native state (cf. [1]). The integration of this kinetic equation in closed form has been given [13]. Using a computer program for calculating the reactivation kinetics of the native tetramer (test B) optimum values for k_1 and k_2 were estimated to be $k_1 = 7.4 \pm 1.4 \times 10^{-4}$ [sec^{-1}] and $k_2 = 1.4 \pm 0.4$ [$mM^{-1}.sec^{-1}$] ** (fig.2, solid lines). In order to fit the profiles of maximum reactivation (test A) the contribution of D* to the total activity was varied for the given values of k_1 and k_2. As shown by the solid lines in fig.1 a residual activity of 50 ± 10% for D* provides satisfactory agreement of the computer data with the observed total reactivation of D* + N. Deviations especially in the initial part of the kinetic traces are caused by the relatively large range of error of the enzymic test at low protein concentrations.

4. Conclusions

Earlier kinetic studies of the reactivation of aldolase led to contradictory results regarding the enzymic activity of the subunits after dissociation of the enzyme in different media [2,6,7]. Based on experiments in a broad range of enzyme concentration the present kinetic analysis proves that the reactivation of aldolase after treatment with guanidine·HCl is in qualitative agreement with that after acid dissociation. Both, maximum reactivation to tetramers and refolded smaller entities on one hand and tetramer formation on the other may be described by the simple model of an irreversible two step consecutive reaction of the uni–bi type, provided that the trypsin-stable intermediate D* shows no more than 50% activity. Full enzymic activity of the isolated subunits in solution is incompatible with the given experimental data.

As in the case of lactic dehydrogenase [14,15] full catalytic function of aldolase requiries association. Whether the second-order rate-determining step belongs to the formation of the dimer or tetramer, i.e., whether the dimer or tetramer is the fully active unit cannot be decided on the basis of the given data.

** A whole spectrum of kinetics following a consecutive uni–bi reaction was calculated by varying both k_1 and k_2. In order to reproduce the kinetics for all concentrations measured, it was found that k_1 and k_2 had to be in a defined range.

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