# Folding and Association of Triose Phosphate Isomerase from Rabbit Muscle

Stefan Zabori, Rainer Rudolph, and Rainer Jaenicke

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-8400 Regensburg

Z. Naturforsch. **35 c**, 999–1004 (1980); received August 7, 1980

Association, Folding, Reconstitution, Triose Phosphate Isomerase

The enzymatic activity and quaternary structure of rabbit muscle triose phosphate isomerase remains unchanged in the concentration range from 2 µg/ml to 2 ng/ml. In this concentration range the enzyme can be reactivated after dissociation and denaturation in 6.5 M guanidine hydrochloride. Removal of the denaturant by dilution and separation of inactive wrong aggregates (5–20%) lead back to active dimers, indistinguishable from the native enzyme as far as enzymatic and physicochemical properties are concerned. Based on the long term stability of the enzyme, the reactivation kinetics were analyzed at low concentrations and 0 °C, conditions where the association of inactive monomers to active dimers is predominant in the process of reactivation. The concentration dependence of the rate of reactivation and the kinetic profiles could be described by a consecutive first-order folding and second-order association reaction scheme with the rate constants $k_{uni} = 1.9 \times 10^{-2}\ s^{-1}$ and $k_{bi} = 3 \times 10^{5}\ M^{-1} \cdot s^{-1}$. This implies that the folded monomers of triose phosphate isomerase, which are intermediate states during reconstitution, cannot possess appreciable enzymatic activity.

## Introduction

The spontaneous structure formation of oligomeric enzymes consists of the consecutive folding and association of the constituent polypeptide chains. Whether or not the isolated folded monomers provide the enzymatically active tertiary structure is still an open question. Detailed investigations using a number of dimeric and tetrameric enzymes have shown that the kinetics of reconstitution may be described by a sequential uni-bimolecular mechanism involving inactive protomers [1]. In the case of tetrameric enzymes the kinetic data do not allow identification of the active entity formed during reactivation. For the dimeric enzymes reactivation can be directly correlated with the formation of the native quaternary structure. Earlier experiments with dimers concentrated on horse-liver alcohol-dehydrogenase [2] and porcine mitochondrial malic dehydrogenase [3]. Since the first example is complicated due to the fact that zinc is required for the catalytic function of the enzyme, malic dehydrogenase represents the only dimeric enzyme investigated in sufficient detail to unequivocally correlate quaternary structure and enzymatic catalysis. As a result, the folded monomeric intermediate has been shown to be inactive; enzyme activity requires association to the dimer. The present paper describes experiments on the reactivation and reassociation of dimeric rabbit muscle triose phosphate isomerase. Since the backbone structure of this enzyme shows distinct differences in comparison with the dehydrogenases apart from general similarities [4] the question arises as to whether or not the enzymatic properties of monomeric intermediates and the reactivation behaviour of the given enzymes were related.

With respect to the reconstitution of triose phosphate isomerase contradicting results have been reported in the literature. While matrix-bound subunits of triose phosphate isomerase seem to indicate full activity of the monomer [5, 6], reconstitution kinetics turn out to obey second order kinetics implying dimer formation as a rate determining step of reactivation and renaturation [7, 8].

In the following study attempts have been made to realize high yields of reactivation and to clarify the refolding pathway of the enzyme under optimum conditions of reconstitution. As shown reactivation and reassociation are coupled reactions obeying a uni-bimolecular kinetic mechanism. The final product of reconstitution is indistinguishable from the native enzyme; the yield of reactivation exceeds 95%.

*Enzyme:* Triose phosphate isomerase (D-glyceraldehyde-3-phosphate ketol isomerase: EC 5.3.1.1) from rabbit muscle.

Reprint requests to Dr. R. Jaenicke.

0341-0382/80/1100-0999 $ 01.00/0

## Materials and Methods

*Materials*

Rabbit muscle triose phosphate isomerase, glycerol-3-phosphate dehydrogenase, D,L-glyceraldehyde-3-phosphate-diethylacetal monobarium salt and NADH were obtained from Boehringer (Mannheim), dithiothreitol from Roth (Karlsruhe). Ultra pure guanidine hydrochloride was supplied by Schwarz/Mann (Orangeburg, N.Y.) and Sephadex G-75 and G-100 from Pharmacia Fine Chemicals (Uppsala). All other reagents were A-grade substances from Merck (Darmstadt); quartz twice-distilled water was used throughout.

*Methods*

*Stock solutions* of the enzyme (~ 1 mg/ml) were prepared by repeated dialysis at 4 °C against 0.1 M triethanolamine buffer, pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol (standard buffer).

*Enzyme concentration* was calculated from $A_{280}^{0.1\%} = 1.31$ cm²/mg [9]; molar concentrations given in the text are based on the molecular weight of the subunit of 26 000.

*Enzyme activity* was determined at 25 °C in standard buffer by a coupled assay with D-glyceraldehyde-3-phosphate as substrate. D,L-glyceraldehyde-3-phosphate was prepared from the diethylacetal by 2 min hydrolysis with 2 N $H_2SO_4$ at 80 °C; the amount of free D-isomer was then determined enzymatically. The test solution contained 2 ng triose phosphate isomerase, 1.4 µmol D-glyceraldehyde-3-phosphate, 0.25 µmol NADH, and 54 µg glycerol-3-phosphate dehydrogenase in a final volume of 1.22 ml. Data are corrected for < 0.01% triose phosphate isomerase activity contained in the given sample of glycerol-3-phosphate dehydrogenase.

*Deactivation and dissociation* of the native enzyme was achieved by 5 min incubation in 6.6 M guanidine hydrochloride at 20 °C. For *reactivation and reassociation*, the denatured enzyme was diluted with standard buffer, pH 7.5, at 0 °C. Care was taken that the amount of guanidine hydrochloride was kept constant at 0.065 M in all reactivation experiments, since it is known that low concentrations of this denaturant may have drastic effects on the kinetics of reactivation of oligomeric enzymes [10].

The *kinetics of reactivation* were analyzed by taking aliquots at defined times after the initiation of reactivation using chilled pipettes. The activity of each aliquot was assayed immediately as described.

*To characterize the reactivated enzyme,* solutions were concentrated in an Amicon diaflo with PM 10 filters; higher aggregates were removed by Millipore filtration and gel filtration on Sephadex G-100 superfine columns. The renatured state was compared to the native and denatured states with respect to enzymatic and physicochemical criteria, such as specific activity, Michaelis constant, intrinsic protein fluorescence (Hitachi Perkin-Elmer MPF 44 spectrophotometer), gel permeation (Sephadex G-75 and G-100), and sedimentation coefficients, as well as molecular weights using the meniscus depletion technique [11]. To estimate sedimentation coefficients in the microgram range, activity transport was measured using fixed partition and moving partition cells (Analytical ultracentrifuge, Beckman Spinco E).

## Results

*Equilibrium studies*

*Native enzyme.* Reconstitution experiments with oligomeric enzymes crucially depend on the long-term stability of the respective systems. The stability of native rabbit muscle triose phosphate isomerase at low concentrations and under solvent conditions used for reactivation had therefore to be tested over the respective time-span. It was in fact found that at concentrations as low as 15 ng/ml, the native enzyme was 96% active after 48 h incubation at 0 °C in standard buffer plus 0.065 M guanidine hydrochloride; extensive incubation (120 h) resulted in less than 20% deactivation. Before analyzing the reconstitution mechanism of oligomeric enzymes, the initial and final states must be characterized, *i.e.* the denatured monomers and the renatured oligomer, which in turn has to be compared to the initial native enzyme. In the case of triose phosphate isomerase, the constant specific activity in the concentration range from 1 to 2000 ng/ml provides cogent evidence that the dimeric structure is unchanged at this dilution, provided that no dissociation into fully active monomers occurs (Fig. 1 a). The latter possibility could be excluded by column chromatography, which showed that in the concentration range from 3 to 2000 ng/ml only active species could be detected belonging to the native dimer with a molecular weight of 52 000 (Fig. 1 a).

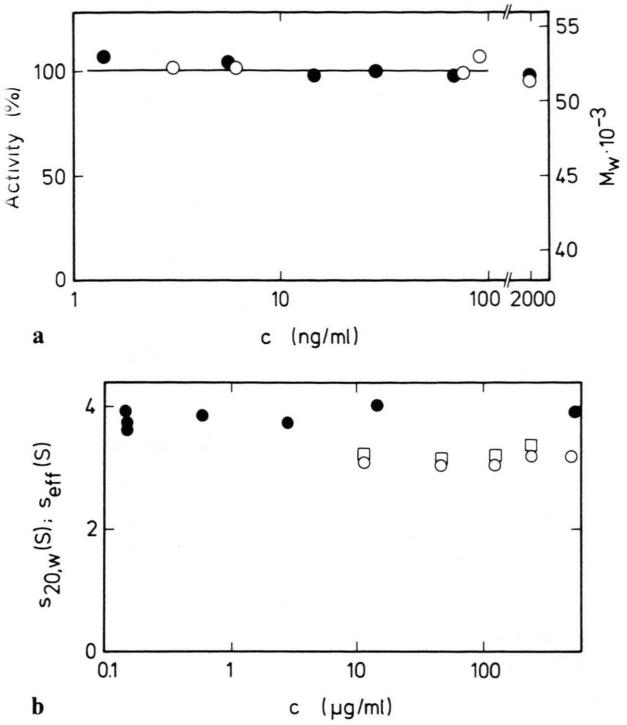


Fig. 1. Influence of enzyme concentration on the specific activity and the quaternary structure of rabbit muscle triose phosphate isomerase. a) Enzymatic activity after 1 h incubation at the given concentrations in standard buffer at 25 °C (●); molecular weight as determined by column chromatography on Sephadex G-100 at 25 °C in standard buffer (○). The column was calibrated using standard proteins of known molecular weight. – b) Sedimentation analysis performed in 0.1 M NaCl + 0.1 M triethanolamine (○) or in 0.02 M Tris (□), both buffer systems at pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol. Sedimentation coefficients at low concentrations determined in standard buffer by activity transport in fixed partition cells (●).

A constant sedimentation coefficient of 3.6 S excludes dissociation in the concentration range from 10 to 500 µg/ml. Sedimentation data obtained by activity transport in fixed partition cells corroborate this finding in a concentration range down to ~ 100 ng/ml (Fig. 1 b).

*Denatured monomers.* It is well established that the present enzyme, like most oligomeric enzymes, can be dissociated into inactive monomers by guanidine hydrochloride denaturation [8, 12]. This could be confirmed for the rabbit muscle enzyme by sedimentation studies in the presence of 6 M guanidine hydrochloride, which gave a sedimentation coefficient of 0.9 ± 0.16 S (Table I). The red shift of 21 nm observed for the intrinsic protein fluorescence upon guanidine dissociation might be taken as an indication for extensive randomization of the isolated chains (Table I).

*Reactivated dimers.* After dissociation and denaturation by 6.5 M guanidine hydrochloride the yield of reactivation is exceedingly high (> 80%), and constant in the concentration range from 20 to 1000 ng/ml (Fig. 2). Lower reactivation yields at lower concentrations might be the result of incomplete reactivation and/or concentration dependent deactivation.

The significance of *in vitro* reassociation experiments depends on the identity of native and reconstituted enzyme. To compare the products of reactivation with native triose phosphate isomerase, the reactivated dimers had to be separated from inactive aggregates as described in the methods section. The reactivated enzyme had the same molecular weight as the native dimers, as determined by comparative column chromatography (Table I). This is confirmed by the sedimentation coefficients which coincide for native and reactivated enzyme within the limits of experimental error (Table I). In addition, both native and reactivated triose phosphate isomerase had similar maxima for the intrinsic protein fluorescence, as well as comparable Michaelis constants for D-glyceraldehyde-3-phosphate (Table I).

### *Kinetic studies*

Reactivation studies have shown that for a variety of dimeric and tetrameric enzymes reactivation necessitates reassociation [1, 13]. For these oligomers reassociation can be studied by analyzing the

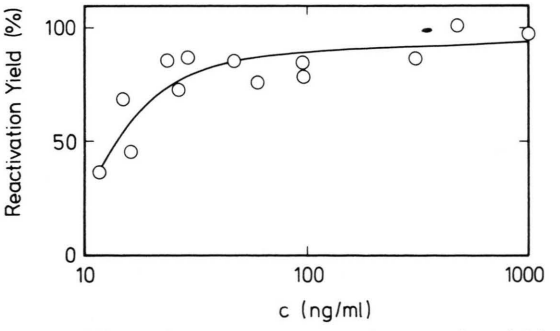


Fig. 2. Effect of enzyme concentration on the yield of reactivation of rabbit muscle triose phosphate isomerase after 5 min deactivation in 6.5 M guanidine hydrochloride at 25 °C. Reactivation at 0 °C by dilution with standard buffer; reactivation time was 144 h.

Table I. Characterization of rabbit muscle triose phosphate isomerase in its native, refolded, and dissociated states.

| State of the enzyme | $s_{20,w}$ [S] | Molecular weight $\times 10^{-3}$ | Activity [%] | $K_M$ [mM] | $\lambda_{max}$ [nm][a] |
|---|---|---|---|---|---|
| Native dimers | 3.60 ± 0.16 | 51.8 ± 1.4 [c] | 100 | 1.8 | 324 |
| Renatured dimers | 3.64 ± 0.16 | 52.0 ± 3.0 [d] | 100 | 1.5 | 322 |
| Denatured monomers[b] | 0.90 ± 0.16 | n. d. [e] | 0 | | 345 |

[a] Maximum of fluorescence emission upon excitation at 285 nm;
[b] denaturation in 6.5 M guanidine hydrochloride;
[c] determined by sedimentation equilibrium;
[d] determined by comparative column chromatography;
[e] n. d., not determined.

reactivation taking place after a certain rate-limiting association reaction. For the dimeric triose phosphate isomerase from rabbit muscle reactivation studies should reveal whether the isolated, but structured monomers, present as intermediates during reconstitution, possess catalytic activity. If they are inactive or only slightly active, reactivation should be of second order in the concentration range where association is slower than the first order folding and reshuffling reactions taking place before and after association. Since triose phosphate isomerase shows high specific activity and stability even at extremes of dilution, the concentration range for kinetic studies could be extended to very low concentrations. To slow down the reassociation rate even further, reactivation was performed at low temperature (0 °C). The respective kinetic traces

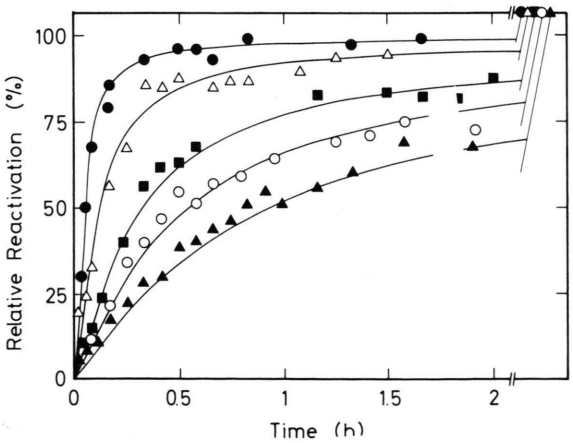


Fig. 3. Kinetics of reactivation of rabbit muscle triose phosphate isomerase after 5 min deactivation in 6.5 M guanidine hydrochloride at 25 °C. Reactivation at 0 °C by dilution with standard buffer at varying enzyme concentrations (nM): ● 39.5; △ 10.5; ■ 3.0; ○ 1.8; ▲ 1.0. Reactivation was calculated relative to final values, determined after 120 h of reactivation. Solid lines are calculated according to an irreversible uni-bimolecular mechanism with $k_{uni} = 1.9 \times 10^{-2}\ s^{-1}$ and $k_{bi} = 3 \times 10^5\ M^{-1} \cdot s^{-1}$.

obtained for reactivation under these conditions in the concentration range from 1 to 40 nM are shown in Fig. 3. A double logarithmic plot of the initial velocities *versus* concentration shows for the lower concentrations the linear dependence with a slope of 2 characteristic for a simple bimolecular rate-limiting process (Fig. 4A). At higher concentrations deviations from the linear behaviour are observed, indicating the influence of first order folding and reshuffling reactions. The rate constant for the association reaction was estimated from the half-times of the reactivation reactions: $k_{bi} = 3 \times 10^5\ M^{-1} \cdot s^{-1}$ (Fig. 4B). Assuming a sequential uni-bimolecular mechanism for the kinetics of reactivation [1], one additional first order rate constant, $k_{uni} = 1.9 \times 10^{-2}\ s^{-1}$, in conjunction with the given $k_{bi}$, is found to be sufficient to describe the observed reactivation relaxations over the whole time course at all enzyme concentrations. The full lines in Fig. 3, which were calculated according to the uni-bimolecular kinetic model, illustrate the quality of the fit. A similar description of the observed reactivation kinetics can be expected from an alternative mechamism comprising second order association followed by a first order folding reaction. A distinction between the two alternatives, as well as other kinetic models proposed previously [7], is not possible on the basis of reactivation data. As shown in the case of lactic dehydrogenase, a discrimination between various kinetic alternatives might be possible by chemical crosslinking during reassociation and subsequent analysis of the association pattern by SDS polyacrylamide gel electrophoresis [14].

Since the experimental setup did not allow the determination of fast reactions, the reconstitution experiments were not extended to higher concentrations, where the deviations from second order kinetics should become prominent, finally approaching first order kinetics.

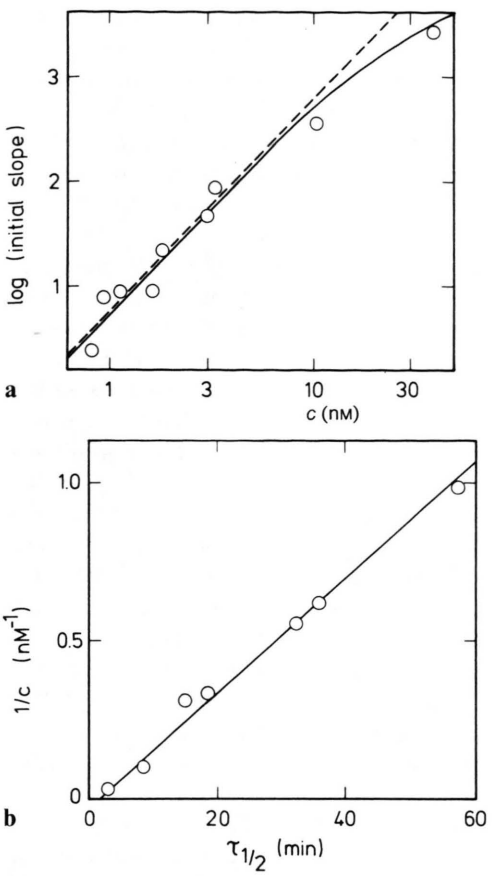


Fig. 4. Reaction order and second order rate constant for the reactivation of rabbit muscle triose phosphate isomerase. Reactivation data are taken from Fig. 3. a) Reaction order of the reactivation calculated from the regain of activity after 5 min. Dotted line: Theoretical line for a simple bimolecular reaction; full line: calculated for an irreversible uni-bimolecular mechanism with the rate constants given in Fig. 3. – b) Second order rate constant determined from the half times of the reactivation relaxations $k_{bi} = 3 \times 10^5\ M^{-1} \cdot s^{-1}$.

## Discussion

The folding pathways of single-chain proteins have been studied in great detail, since the determination of rate-limiting steps and the characterization of intermediates of folding should greatly facilitate the calculation of protein structures from sequence data. The association behaviour of oligomeric enzymes, on the other hand, is far less characterized. Both processes must be coordinated for these systems, since correct association of subunits requires a certain extent of folding of the individual chains which is necessary for their mutual recognition. In a final reshuffling process, occuring after association, the native backbone structure of the active enzyme is formed. If the folded monomeric intermediates of reconstitution show full activity, reactivation must be independent of the subsequent association reaction. If, on the other hand, the structured monomers are inactive, reactivation must approach second-order kinetics at low enzyme concentrations where association becomes rate-limiting in the overall process of reconstitution. Based on this concept, previous investigations with some dimeric and tetrameric dehydrogenases proved the monomers to be enzymatically inactive, no matter whether dissociation was achieved by acidic pH, urea, guanidine hydrochloride [2, 3, 15–17] or high hydrostatic pressure [18]. In all cases the reactivation is characterized by complex kinetics due to the influence of first-order folding reactions besides the obligatory association reaction. It has been found that, to a first approximation, a consecutive uni-bimolecular process is sufficient to describe the reactivation kinetics. In the case of the tetrameric enzymes, reactivation cannot be correlated with a specific association reaction on the mere basis of the reactivation data. For dimeric enzymes, such as triose phosphate isomerase or malic dehydrogenase [3], the reactivation data can be directly compared to the reconstitution process since in both cases only one association step is involved.

The subunits of triose phosphate isomerase are composed of an inner barrel of eight parallel $\beta$-strands, connected by $\alpha$-helices [19]. Although $(\beta\alpha)_2\beta$ structures are found in dehydrogenases as well as in triose phosphate isomerase, the distinct structure of an inner $\beta$-barrel sets triose phosphate isomerase well apart from the dehydrogenases [4]. The intersubunit interactions in triose phosphate isomerase seem to be quite strong, as indicated by the observation that no deactivation or dissociation of the enzyme can be detected upon extensive dilution. Denaturation and dissociation of the enzyme by guanidine hydrochloride is reversible, and the product of reconstitution is indistinguishable from the original native enzyme concerning a variety of enzymatic and physicochemical properties.

Previous reactivation experiments with triose phosphate isomerase gave strong evidence that reassociation is a necessary prerequisite for reactivation [7, 8]. It could be shown that the half-times for

the regain of enzymatic activity of denatured rabbit muscle triose phosphate isomerase after dilution into the assay mixture depended markedly on protein concentration at low concentrations [7]. A more detailed picture could be obtained for the reactivation of chicken muscle triose phosphate isomerase in the range of the dimer-monomer transition [8]; it was concluded that under these conditions renaturation follows second-order/first-order competitive kinetics. Although these kinetic studies imply that isolated monomers of the enzyme should be inactive, contradictory results have been reported for matrix-bound subunits, which seemed to indicate full activity of the monomers [5, 6]. The latter approach contains various potential sources of error, such as multi-point attachment to the matrix or effects of matrix binding on the enzymatic properties of the enzyme. Similar results for matrix bound subunits of lactic dehydrogenase [20] were corrected after careful reexamination with the same experimental technique showing that the isolated monomers are inactive [21]. In the present study the reactivation of rabbit muscle triose phosphate isomerase was analyzed under essentially irreversible conditions, *i.e.* outside the range of the monomer-dimer transition. It has been shown that at low enzyme concentrations a transition from first to second order kinetics occurs; the respective constant for the reassociation reaction at 0 °C could be determined with high accuracy. The concentration dependence of the reaction order, as well as the reactivation relaxations could be adequately described by an irreversible consecutive uni-bimolecular reaction sequence, similar to that determined for the reactivation of certain dehydrogenases [1]. Despite the phenomenological similarities in the kinetic mechanisms a distinct difference is evident comparing the absolute values of the rate constants of reassociation: Reassociation of triose phosphate isomerase at 0 °C is found to be $10^4$ times faster than the rate limiting reassociation of pig muscle lactic dehydrogenase at the respective temperature [22]. Triose phosphate isomerase reassociates with a rate constant close to the value calculated for diffusion controlled association of comparable protomers ranging from $10^5$ to $10^9$ $M^{-1} \cdot s^{-1}$, depending on the respective steric requirements [23–25]. One possible explanation for the fast association of triose phosphate isomerase as compared to the dehydrogenases may be the formation of a loose complex upon association, with unspecific geometrical restrictions. This loose complex would then rearrange to give the native enzyme. It is hoped that differences in the reconstitution mechanism observed for various enzymes might open the way for a better understanding of the correlation of surface structure and subunit assembly. The final aim would be predictions concerning the structure and stability of oligomers in terms of the surface groups involved in the specific inter-subunit contacts [26].

[1] R. Jaenicke and R. Rudolph, Protein Folding (R. Jaenicke, ed.), pp. 525–548, Elsevier/North-Holland Biomedical Press, 1980.
[2] R. Rudolph, J. Gerschitz, and R. Jaenicke, Eur. J. Biochem. **87,** 601–606 (1978).
[3] R. Jaenicke, R. Rudolph, and I. Heider, Biochemistry **18,** 1217–1223 (1979).
[4] D. C. Phillips, M. J. E. Sternberg, J. M. Thornton, and I. A. Wilson, J. Mol. Biol. **119,** 329–351 (1978).
[5] D. A. Fell and C. J. Branford White, Biochem. Biophys. Res. Commun. **67,** 1013–1018 (1975).
[6] C. J. Branford White and D. A. Fell, Trans. Biochem. Soc. **4,** 620–622 (1976).
[7] S. G. Waley, Biochem. J. **135,** 165–172 (1973).
[8] J. D. McVittie, M. P. Esnouf, and A. R. Peacock, Eur. J. Biochem. **81,** 307–315 (1977).
[9] I. L. Norton, P. Pfleiderer, C. D. Stringer, and F. C. Hartmann, Biochemistry **9,** 4952–4958 (1970).
[10] G. Zettlmeißl, R. Rudolph, and R. Jaenicke, Eur. J. Biochem. **100,** 593–598 (1979).
[11] D. A. Yphantis, Biochemistry **3,** 297–317 (1964).
[12] J. D. McVittie, M. P. Esnouf, and A. R. Peacock, Eur. J. Biochem. **29,** 67–73 (1972).
[13] R. Jaenicke, Naturwissenschaften **65,** 569–577 (1978).
[14] R. Hermann, R. Rudolph, and R. Jaenicke, Nature **277,** 243–245 (1979).
[15] R. Rudolph and R. Jaenicke, Eur. J. Biochem. **63,** 409–417 (1976).
[16] R. Rudolph, I. Heider, E. Westhof, and R. Jaenicke, Biochemistry **16,** 3384–3390 (1977)
[17] R. Rudolph, I. Heider, and R. Jaenicke, Eur. J. Biochem. **81,** 563–570 (1977).
[18] B. C. Schade, H.-D. Lüdemann, R. Rudolph, and R. Jaenicke, Biophys. Chem. **11,** 257–263 (1980).
[19] P. W. Banner *et al.*, Nature **255,** 609–614 (1975).
[20] J. C. Cho and H. E. Swaisgood, Biochim. Biophys. Acta **334,** 243–256 (1974).
[21] W. W.-C. Chan and K. Mosbach, Biochemistry **15,** 4215–4222 (1976).
[22] R. Rudolph, I. Heider, and R. Jaenicke, Biochemistry **16,** 5527–5531 (1977).
[23] H. Gutfreund, Enzymes: Physical Principles, p. 159, Wiley-Interscience, London 1972.
[24] H. H. Osborn and M .R. Hollaway, Biochem. J. **143,** 651–662 (1974).
[25] M. Bothwell and H. K. Schachman, Proc. Nat. Acad. Sci. **71,** 3221–3225 (1974).
[26] A. A. Rashin and B. K. Yudman, Molekulyarnaya Biologiya **13,** 698–712 (1979).