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Stadel, J. M., DeLean, A., & Lefkowitz, R. J. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 1-43.
Steer, M. L., & Wood, A. (1979) *J. Biol. Chem. 254*, 10791-10797.
Terasaki, W. L., & Brooker, G. (1978) *J. Biol. Chem. 253*, 5418-5425.
Thomsen, W. J. (1989) Ph.D. Thesis, University of Michigan, Ann Arbor, MI.
Thomsen, W. J., Jacquez, J. A., & Neubig, R. R. (1988) *Mol. Pharmacol. 34*, 814-822.
Tolkovsky, A. M., & Levitzki, A. (1978) *Biochemistry 17*, 3795-3810.
Tolkovsky, A. M., Braun, S., & Levitzki, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 213-217.
Tota, M. R., Kahler, K. R., & Schimerlik, M. I. (1987) *Biochemistry 26*, 8175-8182.
U'Prichard, D. C., Mitrius, J. C., Kahn, D. J., & Perry, B. D. (1983) in *Molecular Pharmacology of Neurotransmitters* (Segawa, T., Yamamura, H. I., & Kuriyama, K., Eds.) pp 53-72, Raven Press, New York.
Valeins, H., Volker, T., Viratelle, O., & Labouesse, J. (1988) *FEBS Lett. 226*, 331-336.
Wagner, R., Ryba, N., & Uhl, R. (1988) *FEBS Lett. 234*, 44-48.

Construction of an Artificial Bifunctional Enzyme, β-Galactosidase/Galactose Dehydrogenase, Exhibiting Efficient Galactose Channeling†

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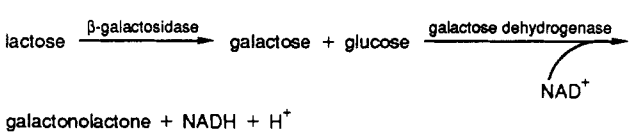
ABSTRACT: The in-frame fusion between two oligomeric enzymes, β-galactosidase and galactose dehydrogenase, is described. The *lacZ* gene was fused to the 3′ end of the *galdh* gene with a linker encoding only three amino acids. The purified artificial bifunctional enzyme displayed the enzymic activity of both gene products. The hybrid protein was found in two major forms, consisting of four and six subunits, but other forms could also be identified. The molecular weight of each subunit was determined to be 145 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The bifunctional enzyme shows kinetic advantages over the identical native system in conversion of lactose to galactonolactone. A higher steady-state rate and a reduction of the transient time are observed. This phenomenon is especially pronounced at low initial substrate concentrations and when the pH is adjusted to a level at which the galactose dehydrogenase activity is much higher than that of the β-galactosidase.

Over the years, we have devoted much attention to the study of the proximity effects of sequentially operating enzymes. Much of this work has been focused on the use of immobilization and cross-linking technologies (Mosbach & Mattiasson, 1970; Srere et al., 1973; Srere & Mosbach, 1974; Månsson et al., 1983). Another approach to the investigation of these effects has been our attempts to prepare artificial bifunctional enzymes by gene fusion. We have previously fused β-galactosidase and galactokinase to form a hybrid protein carrying both activities in order to obtain a better understanding of the microenvironmental effects caused by the proximity of the two catalytic entities (Bülow et al., 1985). The intermediate product, galactose, was more efficiently transferred to the second enzyme in the reaction sequence in an assay medium with a viscosity similar to that found in vivo (Bülow, 1987). These latter studies in a sense also initiate the investigation of the spatial organization of enzymes in relation to each other that has proved to be essential in many metabolic sequences and cycles. However, enzymes of different biological origin which catalyze the same reaction sequence represent a variety of gene arrangements, as illustrated by the presence of individual enzymes, multienzyme complexes, and multifunctional enzymes (Welch, 1985). Therefore, the hypothesis was put forward early that bi- and multifunctional enzymes have evolved through gene fusion (Mowbray & Moses, 1976), a hypothesis that later has been strengthened experimentally through comparison of DNA sequences (Zalkin et al., 1984).

In the present study, we have extended our investigation of artificial bifunctional enzymes to include the design and preparation of a hybrid protein consisting of two naturally occurring oligomeric enzymes. The structural gene of β-galactosidase (*Escherichia coli*), a tetrameric enzyme, was thus ligated to the 3′ end of the gene of galactose dehydrogenase (*Pseudomonas fluorescens*), a dimeric enzyme (Blachnitzky et al., 1974). These enzymes catalyze the sequential hydrolysis of lactose followed by the oxidation of the galactose formed to the corresponding lactone:



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Apart from the theoretical insights gained from the construction of such artificial bifunctional enzymes, they can also be used advantageously in enzyme technology. For instance, the sequential reaction above is of practical importance, since it is frequently utilized for lactose analyses from various biological samples.

Experimental Procedures

Materials

Chemicals and Reagents. The following reagents were purchased from the indicated suppliers: enzymes used for DNA manipulation, β-galactosidase, and galactose dehydrogenase (Boehringer Mannheim); deoxyribonucleotides (dNTPs)[1] (Amersham); acrylamide and bis(acrylamide) (Bio-Rad); Sephacryl S-400 Superfine, FPLC equipment, Mono Q 5/5 column, and kits containing high molecular weight standard proteins for gel filtration and polyacrylamide gel electrophoresis (Pharmacia AB). All other reagents were commercially available and of analytical grade.

Bacterial Strains, Plasmids, and Indicator Plates. E. coli strain F'11 *recA* [(lac, pro) Δthi, rifA, strA, recA/F'lacI^{q}Z$^-$, pro$^+$] (Rüther et al., 1981), which overproduces the *lac* repressor 100-fold, was used as bacterial host in all experiments.

Plasmid pBT 42 has been described earlier (Buckel & Zehelein, 1981). Plasmid pMC 1871 (Shapira et al., 1983) was purchased from Pharmacia AB. *LacZ*$^+$ colonies were identified on indicator plates containing Xgal and ampicillin (Miller, 1972).

Methods

Construction of Plasmids. Restriction enzyme digests and other cloning procedures were performed as described by Maniatis et al. (1982). Plasmids were prepared according to Birnboim and Doly (1979). To obtain a DNA linker encoding the 26 carboxy-terminal amino acids of galactose dehydrogenase without its own translational stop signal, 4 chemically synthesized oligonucleotides were purchased from Pharmacia AB. Information about the DNA sequence of the galactose dehydrogenase gene was kindly supplied by Dr. Sperka prior to publication. These oligonucleotides were phosphorylated with polynucleotide kinase, mixed, heated to 55 °C for 30 min, and then slowly cooled to room temperature to allow hybridization. Furthermore, at this step, a *Bam*HI site was introduced at the 3′ end of the gene to simplify the following ligation to the *lacZ* gene.

Purification of β-Galactosidase/Galactose Dehydrogenase. The fusion protein was purified according to the procedure of Fowler and Zabin (1983) with some minor modifications. The hybrid protein was isolated from *E. coli* F'11 *recA* carrying pDZ 10 grown to late exponential phase in LB broth (Miller, 1972) supplemented with 50 mg/L ampicillin. The cells were harvested by centrifugation at 10000*g* for 10 min and washed once with 0.04 M Tris-HCl, pH 8.0, containing 0.5 mM EDTA, 1 mM $MgCl_2$, and 1 mM DTT (buffer A). After resuspension in the same buffer, bacterial extracts were prepared by sonic disintegration for 10 × 15 s (output 5, Sonifer B-30, Branson Sonic Power) and were clarified by centrifugation at 20000*g* for 30 min. Solid ammonium sulfate was then added slowly to the supernatant until 40% saturation was reached. After the precipitate was pelleted by centrifugation at 20000*g* for 30 min, it was dissolved in buffer A and dialyzed overnight against the same buffer. Any precipitate was removed by centrifugation at 40000*g* for 10 min. These steps were carried out at 0–4 °C. The supernatant of the preceding step was applied to an FPLC-Mono Q 5/5 column previously equilibrated with buffer A containing 0.3 M NaCl. After the column was washed with 8 column volumes, a linear gradient of 0.3–0.4 M NaCl was applied. These steps were carried out at room temperature. Fractions with β-galactosidase and galactose dehydrogenase activities were combined and concentrated by ammonium sulfate precipitation. The protein was dissolved in 0.1 M Tris-HCl, pH 8.0, containing 1 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM DTT. The protein solution was finally subjected to gel filtration on a column of Sephacryl S-400 Superfine at 4 °C.

Enzyme Assays. During purification, β-galactosidase was assayed by hydrolysis of 0.8 g/L ONPG (Miller, 1972) in a buffer consisting of 0.1 M NaH_2PO_4, pH 7.0, and 1 mM $MgSO_4$. One unit of enzyme hydrolyzes 1 μmol of lactose per minute at room temperature. This corresponds to the hydrolysis of 17 μmol of ONPG per minute. Galactose dehydrogenase activity was determined by using 16.6 mM galactose as substrate (Buckel & Zehelein, 1981). One unit of galactose dehydrogenase oxidizes 1 μmol of galactose per minute at room temperature in a buffer consisting of 0.09 M Tris-HCl, pH 8.5, and 0.5 mM NAD.

Heat Stability Measurements. Heat stability measurements of bifunctional enzyme, galactose dehydrogenase, and β-galactosidase were carried out at 50 and 60 °C in 0.1 M Tris-HCl, pH 8.0, containing 1 mM $MgCl_2$ and 1 mM DTT. Bovine serum albumin was added to give a final protein concentration of 1 mg/mL.

Determination of pH Profiles. The β-galactosidase and galactose dehydrogenase activities of the hybrid protein were determined in 0.1 M Tris-HCl in the pH range from 7.0 to 10.5 containing 0.059 M $MgCl_2$. ONPG and galactose were used as substrates for determining the separate activities, and the same assays were performed with the native enzymes. The coupled activity was assayed with lactose as substrate.

Substrate Channeling. In order to monitor any difference in the rates of conversion of lactose to galactonolactone between the fusion protein and the native enzymes, assays were performed in 0.09 M Tris-HCl, pH 7.5 and pH 8.5, containing 0.059 M $MgCl_2$ (buffer B) (Beutler, 1984), 4 mM NAD, and lactose in concentrations of 0.2–20 mM. To match the activities of the native and bifunctional enzymes, the galactose dehydrogenase activity was monitored in buffer B containing 16.6 mM galactose and 4 mM NAD. The β-galactosidase activity was assayed in this case in the same buffer containing 38 mM lactose, 4 mM NAD, and an excess of galactose dehydrogenase. One microliter of bifunctional enzyme solution corresponds to 4.0 milliunits of β-galactosidase and 5.2 milliunits of galactose dehydrogenase at pH 7.5. The separate activities at pH 8.5 are 1.8 and 16 milliunits for β-galactosidase and galactose dehydrogenase, respectively. The appropriate amounts of the hybrid enzyme (10 μL), or the native enzymes, with separate activities equal to those of the fusion protein, were added to the assay solution. The volume of the assay was adjusted to 1 mL with buffer B. The rate of NADH formation was followed spectrophotometrically at 340 nm.

Protein Determination. Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

[1] Abbreviations: dNTPs, deoxyribonucleotides; ONPG, 2-nitrophenyl β-D-galactopyranoside; Xgal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, molecular weight; PRAI-IGPS, *N*-(5-phosphoribosyl)-anthranilate isomerase–indole-3-glycerol-phosphate synthase; K_M, Michaelis constant.

Table I: Purification of the Hybrid Protein from *E. coli* F'11 *recA*/pDZ 10[a]

fraction	volume (mL)	total protein (mg)	β-galactosidase total act. (units)	β-galactosidase sp act. (units/mg)	galactose dehydrogenase total act. (units)	galactose dehydrogenase sp act. (units/mg)
homogenate	26	340	61	0.18	150	0.44
ammonium sulfate 0–40%	3.0	39	37	0.95	110	2.8
FPLC-MonoQ	4.0	2.1	16	7.6	28	13
Sephacryl S-400	17	0.80	13	16	14	17

[a] The results presented are for a purification from 1 L of culture medium grown to late exponential phase. Full details are given under Experimental Procedures.

Electrophoresis in Polyacrylamide Gel. SDS–PAGE was performed on 8% polyacrylamide slab gels using a Tris–glycine, pH 8.3, discontinuous buffer system as described by Laemmli (1970). The molecular weight of the enzyme subunits was determined by comparing their relative mobilities with those of the standard proteins: myosin (M_r 205 000); β-galactosidase (116 000); phosphorylase *b* (97 400); bovine serum albumin (67 000); albumin, egg (45 000); and carbonic anhydrase (29 000).

Polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions was performed on 4, 6, 7.5, and 8% gels using the buffer described above but without SDS. The molecular weights of the enzyme complexes were estimated by comparing their relative mobilities with those of standard proteins followed by the construction of Ferguson plots (Ferguson, 1971), and further treatments of the results were made according to Alder et al. (1975). The standard proteins were thyroglobulin (669 000), β-galactosidase (464 000 and 696 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), and bovine serum albumin (67 000). The standard proteins were detected with Coomassie brilliant blue. To visualize β-galactosidase activity, the gel was stained with Xgal (50 μg/mL). Galactose dehydrogenase activity was detected with 0.1 M Tris-HCl, pH 8.5, containing 30 mM galactose, 0.6 mM NAD, 0.25 mM nitro blue tetrazolium, and 0.013 mM phenazine methosulfate.

Results

Construction of pDZ 10. A schematic representation of the cloning strategy for the construction of the plasmid pDZ 10, encoding an in-frame fusion between the structural genes of β-galactosidase and galactose dehydrogenase, is outlined in Figure 1. pBT 42 (Buckel & Zehelein, 1981) carries the galactose dehydrogenase gene but lacks any suitable cloning sites in the 3′ end. Therefore, the *Sal*I site in the noncoding region was first removed by digesting the plasmid partially with *Sal*I followed by treatment with Klenow fragment. A DNA fragment encoding the 26 carboxy-terminal amino acids of galactose dehydrogenase was thereafter inserted between the unique *Sal*I and *Cla*I sites of pDH 100. The obtained plasmid, pDH 101, harbors the entire galactose dehydrogenase gene with a *Bam*HI site convenient for cloning of the *lacZ* gene from pMC 1871. Thus, pMC 1871 was digested with *Bam*HI, and the *lacZ* fragment was inserted into pDH 101. When transformed into *E. coli* F'11 *recA*, selection on indicator plates yielded *lacZ*$^+$ colonies which were screened for plasmids with a size of 9000 bp. One of the isolated plasmids, pDZ 10, encodes an in-frame fusion between β-galactosidase and galactose dehydrogenase which codes for a single polypeptide of 1322 amino acid residues.

Purification of β-Galactosidase/Galactose Dehydrogenase. The fusion protein could be purified according to a previously described procedure for the hybrid protein β-galactosidase/galactokinase (Bülow, 1987). A summary of the purification steps is presented in Table I. The elution profiles from ion-

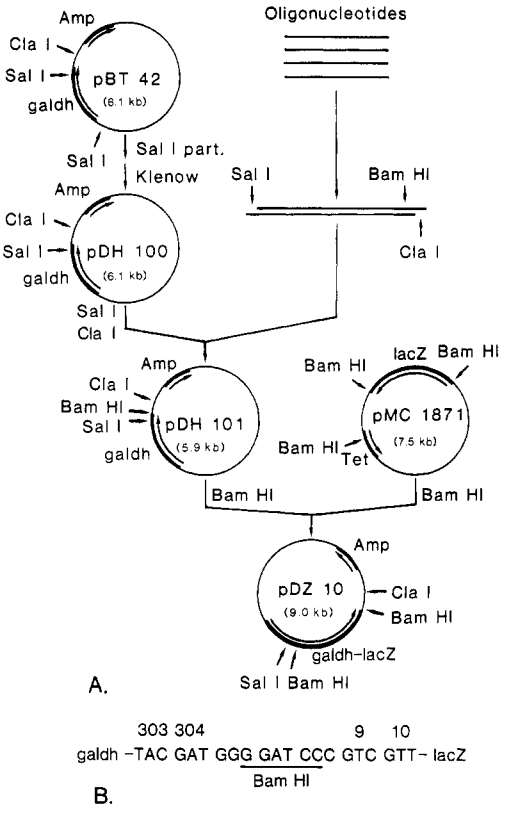

FIGURE 1: (A) Schematic representation of the construction of the chimeric plasmid pDZ 10 coding for an in-frame fusion between galactose dehydrogenase (*galdh*) and β-galactosidase (*lacZ*). (B) Sequence of nucleotides of the linker region between the fused galactose dehydrogenase (*galdh*) and β-galactosidase (*lacZ*) genes. The numbers indicate the amino acid residue numbers of the galactose dehydrogenase and β-galactosidase, respectively.

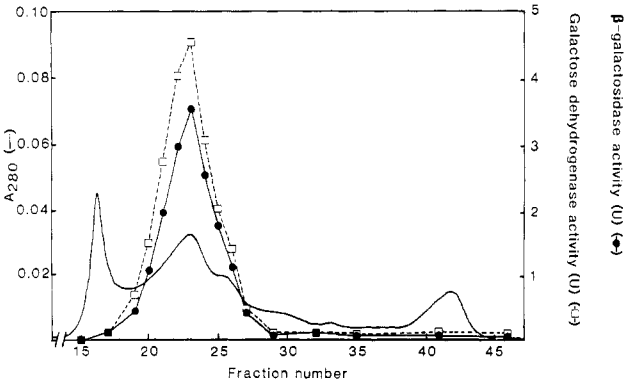

FIGURE 2: Gel filtration chromatography on Sephacryl S-400 Superfine of the bifunctional enzyme. Fraction volume: 3.5 mL

exchange chromatography on FPLC-MonoQ and gel filtration on Sephacryl S-400 Superfine (Figure 2) showed that the two enzyme activities copurified. Further evidence for the presence of a single hybrid protein, carrying both activities, was given

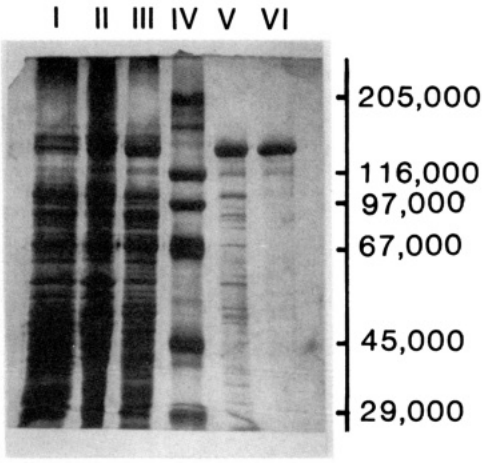

FIGURE 3: Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of the hybrid protein. The protein bands were visualized by silver staining. (I) Whole cells; (II) after sonication; (III) after ammonium sulfate precipitation; (IV) molecular weight standards myosin (M_r 205 000), β-galactosidase (116 000), phosphorylase *b* (97 400), bovine serum albumin (67 000), albumin, egg (45 000), and carbonic anhydrase (29 000); (V) after FPLC-Mono Q column; (VI) after gel filtration on Sephacryl S-400 Superfine.

by an activity staining procedure after polyacrylamide gel electrophoresis under nondenaturing conditions. The fusion protein showed a lower electrophoretic mobility than both native β-galactosidase and galactose dehydrogenase. However, the two enzymic activities of the hybrid enzyme were found superimposed. As judged from SDS–PAGE (Figure 3), the hybrid protein was at least 95% pure after the last purification step. The bifunctional enzyme had specific activities of 16 units/mg for β-galactosidase and 17 units/mg for galactose dehydrogenase. When corrected for the increase of molecular weight caused by the gene fusion, these specific activities correspond to 90–100% and 40–50% for native β-galactosidase and galactose dehydrogenase, respectively.

Molecular Weight Determination. The molecular weight of the fusion protein was determined by gel filtration and polyacrylamide gel electrophoresis followed by activity staining. In the gel filtration experiment, the protein eluted in two peaks corresponding to a molecular weight of 890 000 and 580 000, respectively. On the activity-stained polyacrylamide gel, two major bands of 840 000 and 530 000 could be seen. As estimated from SDS–PAGE, the subunit weight of the hybrid protein is 145 000. Galactose dehydrogenase is a dimeric enzyme with a determined M_r = 33 000 for each monomer (Sperka et al., personal communication) while β-galactosidase is a tetrameric enzyme with a monomer molecular weight of 116 000 (Kalnins et al., 1983). The theoretical value of the subunit of the hybrid protein is 148 300 when corrected for the linker region and the missing first eight nonessential amino acid residues of β-galactosidase. These data suggest that the fusion protein is mainly present in two forms, a tetrameric and a hexameric form. The gel filtration experiments showed that the β-galactosidase hybrid was, in this case, present to a higher extent as hexamer (Figure 2). Previously, native β-galactosidase (Marchesi et al., 1969) and hybrid β-galactosidase fusion proteins (Bülow, 1987; Fowler & Zabin, 1983) have also been observed in the tetrameric, hexameric, and octameric forms, with a strong preference for the tetrameric form.

Thermostability. To determine any difference in stability against thermal denaturation, both the bifunctional enzyme and the native enzymes were heated to 50 and 60 °C (Figure 4). The bifunctional enzyme retained only 2% of the β-galactosidase activity after incubation at 50 °C for 30 min, while native β-galactosidase is unaffected at this temperature. However, the native β-galactosidase is denatured quickly at higher temperatures. For instance, it is inactivated in less than 5 min at 60 °C. In contrast, native galactose dehydrogenase was less stable to heat than the galactose dehydrogenase part of the bifunctional enzyme. As shown in Figure 4, the galactose dehydrogenase moiety of the hybrid protein retained 40% of the original activity after incubation at 60 °C for 30 min while native galactose dehydrogenase only retained 20% of the activity after 5 min at 60 °C. After the heat incubation, those samples were also applied to polyacrylamide gel electrophoresis, and activity staining of the gels showed that the hybrid protein, with tetrameric and hexameric aggregation, had dissociated to complexes consisting of dimers. Thus, β-galactosidase is enzymatically active only in the tetrameric and hexameric forms (Fowler & Zabin, 1983) (Figure 6). On prolonged storage at 4 °C, the galactose dehydrogenase moiety of the hybrid protein gradually loses its activity while the β-galactosidase activity remained stable. When this "old" hybrid enzyme preparation was heated to 50 °C, there was an increase in galactose dehydrogenase activity that strictly followed the decrease in β-galactosidase activity (Figure 5).

Determination of pH Profiles. In the pH range tested, the maximal activity for the β-galactosidase moiety of the fusion enzyme was found at pH 7.0, the same as for the native enzyme. The galactose dehydrogenase part exhibits a slight shift in pH optimum, showing the highest activity at pH 10.5, while native galactose dehydrogenase showed a pH optimum of 10.0. In the coupled reaction, a pH optimum of 7.0 was observed.

Substrate Channeling. In order to investigate the proximity effects and the possibilities of substrate channeling, an assay solution consisting of either the bifunctional enzyme, taken after the FPLC purification step, or a comparable system of the two native separate enzymes was employed, as described under Experimental Procedures. The steady-state rates of NADH formation by the bifunctional and the native system are presented in Tables II and III. The results indicated that the genetically fused enzymes exhibited an overall higher steady-state rate, in comparison with the system of separate enzymes, in the sequential reaction that converts lactose to

Table II: Kinetic Parameters Obtained at pH 8.5 for the Bifunctional and Native Enzymes, Respectively

	lactose concn (mM)		
enzyme system	20	2	0.2
(A) Rates of NADH Formation (nmol/min) at Steady State			
bifunctional enzyme	17	3.7	0.46
native enzymes	11	1.8	0.19
bifunctional/native	1.5	2.1	2.4
(B) Transient Time, τ (min)			
bifunctional enzyme	0.35	0.60	4.0
native enzymes	1.3	4.4	17
bifunctional/native	0.27	0.14	0.24

Table III: Kinetic Parameters Obtained at pH 7.5 for the Bifunctional and Native Enzymes, Respectively

	lactose concn (mM)		
enzyme system	20	2	0.2
(A) Rates of NADH Formation (nmol/min) at Steady State			
bifunctional enzyme	30	14	3.1
native enzymes	35	14	2.3
bifunctional/native	0.86	1.0	1.35
(B) Transient Time, τ (min)			
bifunctional enzyme	0.75	1.3	1.4
native enzymes	1.2	2.0	2.5
bifunctional/native	0.62	0.65	0.56

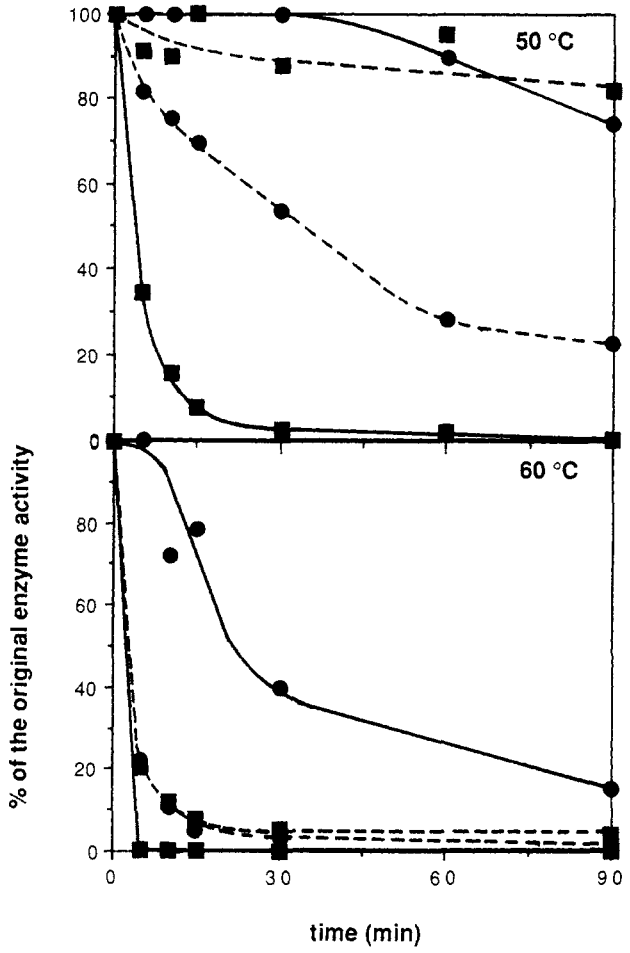

FIGURE 4: Heat stability of the bifunctional enzyme compared with native enzymes. The protein solutions were incubated at 50 and 60 °C for the indicated length of time, and residual activities were determined. (■---■) Native β-galactosidase; (■—■) hybrid β-galactosidase; (●---●) native galactose dehydrogenase; (●—●) hybrid galactose dehydrogenase.

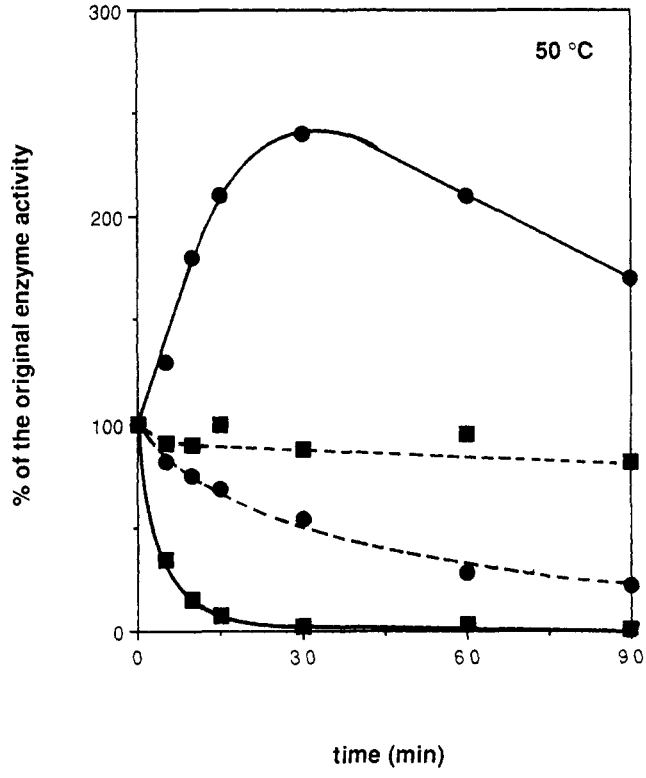

FIGURE 5: Heat stability of bifunctional enzyme that had been subjected to storage at 4 °C for 2 weeks in buffer A. The protein solutions were incubated at 50 °C for the indicated length of time, and residual activities were determined. (■---■) Native β-galactosidase; (■—■) hybrid β-galactosidase; (●---●) native galactose dehydrogenase; (●—●) hybrid galactose dehydrogenase.

galactonolactone. At pH 8.5, with lower concentrations of lactose (0.2 mM), the bifunctional enzyme had an apparent steady-state rate of more than twice the steady-state rate of the native enzymes, in the coupled reaction. Furthermore, the time before steady-state conditions were reached, the transient time (Friedrich, 1984), for the coupled reaction was markedly shorter with the fused enzyme than with the native ones (Table II). A difference in galactose channeling at pH 7.5 between the two systems could only be observed with the lowest lactose concentration (0.2 mM). With this substrate concentration, the bifunctional enzyme had an overall reaction rate that was 35% higher than that of the native enzymes. Furthermore, the difference in transient time was not as pronounced at this pH as it was at pH 8.5 (Table III).

Discussion

Upon ligation of the structural genes of two oligomeric enzymes, the formation of a three-dimensional protein network in vivo could be expected due to the interactions between the protein monomers. Through the different steps of purification, no enzymic activity was thus associated with insoluble particles. Even though the active form of the prepared artificial bifunctional enzyme proved to be perfectly soluble, there is a potential of formation of larger aggregates in vivo. As judged from gel filtration and polyacrylamide gel electrophoresis under nondenaturing conditions, followed by activity staining, the active forms of enzyme aggregation appear to consist mainly of tetramers (1/3) and hexamers (2/3) where each β-galactosidase monomer is carrying a galactose dehydrogenase monomer. This latter aggregation can be looked upon as the formation of a partial protein network since β-galactosidase is normally found predominantly as a tetramer. Due to the dimeric nature of galactose dehydrogenase, it seems plausible that these monomers are able to interact with each other within this core unit in both forms of aggregation. However, at the present stage, it cannot be excluded that the monomers exhibit galactose dehydrogenase activity in the fused state. Fusion obviously interferes with the normal noncovalent interactions between the galactose dehydrogenase monomers. The thermal denaturation experiments with enzyme that has been subjected to storage at 4 °C indicate that a more favorable galactose dehydrogenase dimer is re-formed only after the β-galactosidase tetra- or hexamer of this "old" enzyme has been disrupted or distorted, since there appears to be a strict correlation between deactivation of the β-galactosidase activity and gain of galactose dehydrogenase activity. The galactose dehydrogenase activity of the "old" enzyme could thus be almost restored to its original activity upon deactivation of the β-galactosidase part of the hybrid.

The length of the connecting segment between the two catalytic moieties might be critical in this respect. In the described construction, this linker region consists of only three amino acid residues. The introduction of a flexible "hinge" region could assist the formation of the correct galactose dehydrogenase monomer–monomer interactions, and additionally, it might give further evidence to the proposed subunit interaction model.

An important consideration in the design of bifunctional enzymes is the orientation of the active centers in relation to each other. To date, the three-dimensional structures of only

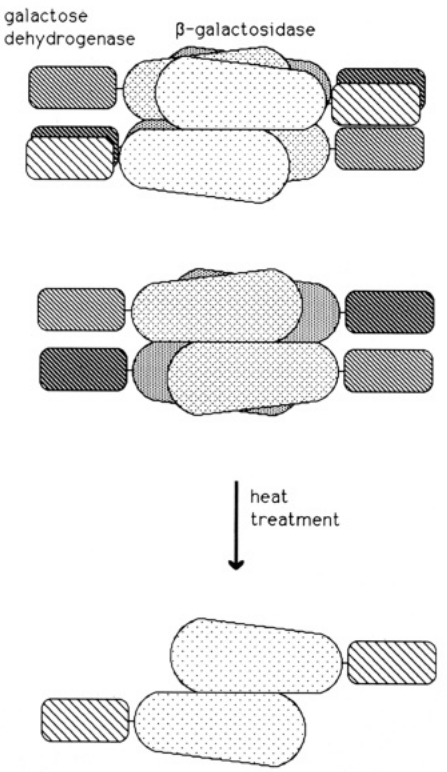

FIGURE 6: Schematic configuration of the proposed forms of the β-galactosidase/galactose dehydrogenase enzyme complexes. The striped and dotted parts represent galactose dehydrogenase monomers and β-galactosidase monomers, respectively. The fusion enzyme consists mainly of hexamers and tetramers which dissociate into dimers when subjected to heat. These dimers show only galactose dehydrogenase activity.

two naturally occurring bifunctional enzymes, Klenow fragment of DNA polymerase I (Ollis et al., 1985) and *N*-(5-phosphoribosyl)-anthranilate isomerase–indole-3-glycerol-phosphate synthase (PRAI–IGPS) (*E. coli*) (Priestl et al., 1987), have been elucidated. In the former case, the active sites seem to be perfectly oriented to perform the sequential reaction. However, in PRAI–IGPS, the active centers appear to operate independently. Therefore, there must have been some other driving forces behind the evolution of this bifunctional protein (Kirschner & Bisswanger, 1976), for instance, simultaneous gene regulation. Since the X-ray structure of neither β-galactosidase nor galactose dehydrogenase is known, an indication of the proximity or potential juxtaposition can be obtained only from kinetic measurements. Thus, the channeling of the formed intermediate, galactose, under steady-state conditions, and also the lag phase before steady-state conditions are reached, the transient time, were determined. In the prepared hybrid protein, a distinct difference in transient time and unexpectedly also in steady-state rate was indeed observed as compared to an identical system composed of native enzymes. This phenomenon became especially pronounced at low initial substrate concentrations and when the pH was chosen such that the galactose dehydrogenase activity was much higher than the β-galactosidase activity. At pH 7.5, with a ratio of galactose dehydrogenase to β-galactosidase activities of 6:5, channeling could thus only be observed with lactose concentrations of 0.2 mM and lower (Table III). At this pH, the apparent K_M for lactose was 1.9 mM for the fused system and 3.4 mM for the system with native enzymes. However, at pH 8.5, with the ratio of activities of galactose dehydrogenase to β-galactosidase of 8:1, substrate channeling became more pronounced (Table II). Furthermore, the apparent K_M for lactose was determined as 11 mM for the fused system and 27 mM for the system with the native enzymes. The pH could thus be used to control the degree of channeling. The ratio between the two activities was lowered when the fused enzyme was subjected to storage at 4 °C. Especially at pH 7.5, this "old" enzyme preparation exhibits a markedly lower substrate channeling. This fact can be explained by the formation of an unfavorable ratio between the galactose dehydrogenase and β-galactosidase activities. Thus, the intermediate substrate, galactose, is in this case probably able to diffuse out of the protein microenvironment.

From a practical point of view, the utilization of hybrid bifunctional enzymes in enzyme technology offers many advantages. Their purification is simplified since only the affinity of one moiety is required during a chromatographic procedure. The galactose dehydrogenase could thus be efficiently purified by taking advantage of the very strong β-galactosidase adsorption to Mono Q. Furthermore, such "ready-made" enzyme sequences should facilitate their application in analysis using biosensors.

References

Alder, S. P., Purich, P., & Stadtman, E. R. (1975) *J. Biol. Chem. 250*, 6264–6272.

Beutler, H.-O. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Bergmeyer, J., & Grassl, M., Eds.) Vol. VI, pp 104–112, Verlag Chemie GmbH, Weinheim.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Blachnitzky, E. O., Wengenmayer, F. A., & Kurtz, G. (1974) *Eur. J. Biochem. 47*, 235–250.

Bradford, M. (1976) *Anal. Biochem. 22*, 248–254.

Buckel, P., & Zehelein, E. (1981) *Gene 16*, 149–159.

Bülow, L. (1987) *Eur. J. Biochem. 163*, 443–448.

Bülow, L., & Mosbach, K. (1987) *Ann. N.Y. Acad. Sci. 501*, 44–49.

Bülow, L., Ljungcrantz, P., & Mosbach, K. (1985) *Bio/Technology 3*, 821–823.

Ferguson, K. A. (1964) *Metabolism 13*, 985–1002.

Fowler, A. V., & Zabin, I. (1983) *J. Biol. Chem. 258*, 14354–14358.

Friedrich, P. (1984) *Supramolecular enzyme organization*, Pergamon Press, Oxford.

Kalnins, A., Otto, K., Rüther, U., & Müller-Hill, B. (1983) *EMBO J. 2*, 592–593.

Kirschner, K., & Bisswanger, H. (1976) *Annu. Rev. Biochem. 45*, 143–166.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning. A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Månsson, M.-O., Siegbahn, N., & Mosbach, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1487–1491.

Marchesi, S. L., Steers, E., & Shifrin, S. (1969) *Biochim. Biophys. Acta 181*, 20–34.

Miller, J. H. (1972) *Experiments in molecular genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mosbach, K., & Mattiasson, B. (1970) *Acta Chem. Scand. 24*, 2093–2100.

Mowbray, J., & Moses, V. (1976) *Eur. J. Biochem. 66*, 25–36.

Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature (London) 313*, 762–766.

Priestle, J. P., Grüther, M. G., White, J. L., Vincent, M. G., Kania, M., Wilson, E., Jardetzky, T. S., Kirschner, K., & Jansonius, J. N. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5690–5694.

Rüther, U., Koenen, M., Otto, K., & Müller-Hill, B. (1981) *Nucleic Acids Res. 9*, 4087–4097.
Shapira, S. K., Chou, J., Richaud, F. V., & Casadaban, M. J. (1983) *Gene 25*, 71–82.
Srere, P. A., & Mosbach, K. (1974) *Annu. Rev. Microbiol. 28*, 61–83.
Srere, P. A., Mattiasson, B., & Mosbach, K. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2534–2538.
Welch, G. R., Ed. (1985) in *Organized Multienzyme Systems*, Academic Press, New York.
Zalkin, H., Paluh, J. L., van Cleemput, M., Moye, W., & Yanofsky, C. (1984) *J. Biol. Chem. 259*, 3985–3992.

A Dynamic Model for the Structure of Acyl Carrier Protein in Solution†

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ABSTRACT: The determination of solution structures of proteins using two-dimensional NMR data is commonly based on the assumption that the structure can be represented by a single rigid conformer. We present here a procedure whereby this assumption can be relaxed and illustrate its application to acyl carrier protein from *Escherichia coli*, a small negatively charged protein with no internal disulfide bonds. The methodology rests on a model having two distinct conformers in dynamic equilibrium. Use of this two-state model results in a dramatic improvement in fit to cross-relaxation-derived distance constraints and a substantial lowering of molecular mechanics energies for individual conformers of acyl carrier protein. The two-state model retains the three-helix motif previously identified on the basis of a one-state structure, but substantial motion of loop regions and the C-terminal peptide, as well as partial disruption of the second helix, is suggested to occur. Support for the existence of these motions can be found in amide exchange rate and spin relaxation time data.

Recent advances in NMR instrumentation and methodology have made it possible to acquire sufficient distance constraints from proton–proton nuclear Overhauser effects (NOEs)[1] to attempt determination of the structures of small proteins in solution (Braun et al., 1983; Havel & Wuthrich, 1985; Clore et al., 1987a; Holak et al., 1988a; Moore et al., 1988). A number of examples of successful application now exist including ones showing excellent agreement of X-ray and NMR structures (Clore et al., 1986, 1987a; Wagner et al., 1987; Kline et al., 1988).

The protocols developed for converting NOE measurements into structures vary widely (Braun & Go, 1985; Clore et al., 1985, 1987b; Wagner et al., 1987; Holak et al., 1988a; Kline et al., 1988). However, most employ a $1/r^6$ interproton distance dependence of NOEs—a relationship that stems from an assumption that protons are rigidly fixed in a well-defined protein structure and that proton–proton dipolar interactions are modulated by a single isotropic molecular tumbling motion. Violation of this assumption by the presence of some types of motion, for example, the presence of rapid, uncorrelated internal motions, will have small effects on derived structures (LeMaster et al., 1988). However, one would expect more severe effects due to the presence of slower internal motions. Recognition of possible violations of assumptions underlying a straightforward conversion of NOE intensities to distances is, in part, reflected in the reluctance of many authors to do more than set, upper and lower bounds, for NOE-based distances (Braun & Go, 1985; Clore et al., 1987b; Wagner et al., 1987). It is possible, however, to employ a more precise distance specification if one explicitly allows for the presence of slow internal motions in deriving a structure. We hope to illustrate this point, with an application to acyl carrier protein from *Escherichia coli* (ACP).

ACP is typical of many proteins studied by NMR in terms of its size, 8847 daltons, and in terms of the quality of NMR data obtained. ACP differs, however, from many proteins studied in that it has no intramolecular disulfide bonds to stabilize a single well-defined structure (Wagner et al., 1987) and in that it may be further destabilized by a high net negative charge (−16 at pH 5.9). It is a protein in which one might expect effects of motion to be pronounced.

The structure of ACP has been determined without explicit treatment of motion, using both a simulated annealing approach, which employs upper and lower bounds for distances (Holak et al., 1988b), and a molecular mechanics pseudoenergy approach, which employs a single best estimate of interproton distances (Holak et al., 1988a). The quality of these structures is often assessed on the basis of convergence of solutions from multiple starting points to a single structure, and on the basis of divergence of interproton distances in the final structures from experimental constraints. The structure produced by simulated annealing looks good on both of these counts. The average RMS deviation of backbone atoms from their mean position is 2 Å, and the number of distance violations greater than 0.5 Å for the average structure is very small. A refinement published during preparation of this paper, in fact, finds a much smaller RMS deviation of back-

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[1] Abbreviations: NOE, nuclear Overhauser effect; ACP, acyl carrier protein; RMS, root mean square; AMBER, assisted model building with energy refinement.

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