Preparation of Hydrocarbon-Adhering Derivatives of Water-Soluble Enzymes

Only a small fraction of an enzyme molecule is involved in substrate binding and catalysis; the remainder may function by influencing interactions between the active site and the environment. Chemical modification might better adapt an enzyme for functioning in a chosen environment. We have produced derivatives of water-soluble enzymes which adhere to hydrocarbon-water interfaces. Similar derivatives may prove valuable in applications such as the enzyme-catalyzed oxidation of petroleum to water-soluble products.

Materials and methods. DDI brand Diisocyanate 1410, a 36 carbon saturated aliphatic hydrocarbon with 2 terminal isocyanate groups was obtained from General Mills Chemicals, Inc. Hexamethylene diisocyanate (1,6-diisocyanatohexane, HMDIC) was obtained from Aldrich Chemicals; hen egg white lysozyme (Sigma Grade I), bovine pancreatic ribonuclease (Type I-A), wheat germ lipase (Type I) and hog pancreatic lipase (steapsin, crude) from Sigma Chemical Co.; olive oil (Best, U.S.P., Lot 714498) from Fisher Scientific Co.; phenylmethylsulfonylfluoride (B Grade) from Calbiochem. Other chemicals used were Reagent or Certified Spectroanalyzed grade.

Dry commerical enzymes were dissolved in aqueous buffers to a concentration of 0.3–1.0 mg/ml. Phenylmethylsulfonylfluoride was added (to 0.001 *M*) to the lipases to protect them from proteolytic contaminants. The diisocyanates were diluted to 0.01 *M* in hydrocarbon (usually n-heptane). The volume ratio of organic to aqueous phase in the reaction mixture was 0.10 ml to 3.0 ml. The ratio of reagent to protein was 100–200 μmoles/g protein. In order to speed reaction between the diisocyanate (organic phase) and protein (aqueous phase), the interface area was increased by 30 sec ultrasonic vibration at 0°C. The resulting emulsion was stirred at room temperature for 3 h, and then centrifuged at $145k \times g$ (average force) for 20 min at 4° or 22°C in order to measure the distribution of protein between the aqueous and emulsion phases. The plastic centrifuge tubes were then punctured at the bottom to allow collection of both phases for protein and enzyme assays. If a film of undissolved material collected at the top of the liquid during centrifugation, this fraction was resuspended in aqueous buffer for measurement.

The rate of reaction between the isocyanate and protein primary amino groups (measured by ninhydrin color yield) was much faster than adsorption of protein to the organic interface. In reactions subjected to rate measurements, adsorption was essentially completed by 3 h of stirring at room temperature.

Results and discussion. Typical protein recovery data for lysozyme treated by this procedure is shown in Table I. While n-heptane alone caused loss of 44% of the lysozyme from the aqueous phase, 64% of the hexamethylene diisocyanate-reacted, and 77% of the DDI-reacted lysozyme were removed from the aqueous phase. Catalytic activity data are not reported since aliphatic hydrocarbon binding inhibits lysozyme[1].

Activity and protein recovery in 2 experiments with ribonuclease are shown in Table II. Deproteinized, dialyzed yeast nucleic acid was used as substrate[2]. In experiment B cytidine-2′(3′)-monophosphate (10 moles/mole RNase), a competitive inhibitor, was used in an attempt to improve the activity yield of the modified enzyme. Removal of protein from the aqueous phase was extensive (51%, 63%) in the DDI-RNase reaction mixture, significant (25%, 34%) in the hexamethylene diisocyanate-RNase reaction mixture and slight with the heptane-RNase control mixture (4% 13%). Catalytic activity recoveries of the modified enzyme were all low (53% to

Table I. Protein recoveries in aqueous phase from lysozyme treated with n-heptane and aliphatic diisocyanates

Rxn.	n-Heptane (λ)	Reagent (λ)	Protein recovery in aqueous phase (%)
I	75	—	56
II	65	10 DDI	23
III	65	10 HMDIC	36

Three ml volumes[3] of lysozyme (0.3 mg/ml) in 0.01 *M* sodium borate, pH 9.0, were treated with (I) n-heptane alone, (II) DDI (0.01 *M*) in n-heptane and (III) hexamethylene diisocyanate (0.01 *M*) in n-heptane. Emulsion was formed by ultrasonic vibration and separated by ultracentrifugation. Protein concentration was measured by A_{280} on Beckman DU.

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Table II. Protein and activity recoveries in aqueous phase from ribonuclease treated with n-heptane and aliphatic diisocyanates

Rxn.	n-Heptane (λ)			Reagents (λ)		
I	75			—		
II	50			25 DDI		
III	50			25 HMDIC		
	A			B		
	I	II	III	I	II	III
Activity recovery in Rxn. mixture (%)	92	53	54	97	57	62
Activity recovery in aqueous phase (%)	95	55	48	97	54	45
Protein recovery in aqueous phase (%)	96	49	75	87	37	66

Three ml volumes[3] of ribonuclease (0.5 mg/ml) in 0.01 *M* sodium borate, pH 9.0, were treated with (I) n-heptane alone, (II) DDI (0.01 *M*) in n-heptane and (III) hexamethylene diisocyanate (0.01 *M*) in n-heptane in the manner noted in Table I. Activity measurements were made with purified yeast nucleic acids[2]. Protein concentrations were measured by A_{280} in experiment A. In experiment B, cytidine-2′(3′)-monophosphate (10 moles/*M* RNase) was included in the rxn. mixture. Protein concentration was measured by biuret-phenol color reaction.

Table III. Activity recoveries and distribution from lipases treated with olive oil and DDI diisocyanate
A) Wheat germ

Sample	Activity recoveries[b]					
	I (%)	(U/ml)[a]	II (%)	(U/ml)[a]	III (%)	(U/ml)[a]
Initial aqueous solution	100	116				
Reaction mixture	43	50	37	43	38	44
Aqueous phase	34	52	34	53	27	42
Interface region (0.5 ml)	9	41	8	37	6	28
Resuspended interface film	—	—	1		4	
Total recovery in fractions	43		43		37	

B) Hog pancreatic

Sample	Activity recoveries[b]					
	I (%)	(U/ml)[a]	II (%)	(U/ml)[a]	III (%)	(U/ml)[a]
Initial aqueous solution	100	28				
Reaction mixture	67	19	124	34	77	22
Aqueous phase	81	23	33	14	14	6
Interface region (1 ml)			46	39	76	64
Resuspended interface film			23		6	
Total recovery in fractions	81		102		96	

Three ml volumes[3] of lipases (0.5 mg/ml in 0.01 *M* potassium phosphate buffer, pH 8.0, plus 0.001 *M* phenylmethylsulfonylfluoride were treated with (I) no additions, (II) 0.1 ml olive oil alone, and (III) DDI in olive oil (0.1 ml), in the manner noted in Table I. Kinetic activity measurements were made in a pH stat (Radiometer), measuring rate of acid release from triacetin by wheat germ lipase A). and from olive oil emulsion by hog pancreatic lipase B((Worthington Biochemical Corp. assay). [a] Concentration of active enzyme. [b] Percent of total activity of original aqueous solution.

62%) compared to the control (92%, 97%). The competitive inhibitor protected the enzyme only slightly against activity loss. Most activity was in the aqueous phase after separating the emulsion.

The possibility that activity losses were due to inaccessability of the active site of the hydrocarbon-adsorbed enzyme to the water-soluble substrate, rather than to denaturation by the chemical modification, was examined by comparing the effect of DDI treatment on recovery and distribution of activity from two functionally similar enzymes, one with a water-soluble and the other with a hydrocarbon-soluble substrate. Table III shows activity recoveries of wheat germ lipase (3a) with water-soluble triacetin substrate and hog pancreatic lipase (3b) with olive oil (hydrocarbon-soluble) substrate. The DDI reagent showed little effect on the recovery of activity of wheat germ lipase with water-soluble substrate. The interface region exhibited lower effective concentration. The catalytic activity of hog pancreatic lipase, on the other hand, was concentrated (140% of initial concentration) in the interface region by the water-insoluble substrate (olive oil) alone[3]. A still more extensive concentration in the interface region was seen with the DDI-derivative (168% of the activity concentration found with the unmodified enzyme subjected to the same conditions, 235% of the initial concentration).

The DDI-enhanced transfer of protein to the hydrocarbon interface region results in a very stable interaction. The DDI-modified proteins are insoluble in aqueous buffer. Equilibrating the olive oil emulsion region containing hog pancreatic lipase with fresh aqueous buffer resulted in significant transfer of catalytic activity back into the aqueous phase from unreacted protein, but little or no catalytic activity could be returned to the aqueous phase from the DDI-reacted protein in the interface region.

These data indicate the feasibility of tailoring enzymes to function in chosen environments. The addition of lipophilic side chains to enzymes greatly enhances their tendency to adhere to water-hydrocarbon interfaces. An enzyme catalyzing hydrolysis of a water-insoluble substrate was hindered only slightly in catalytic function by this chemical modification[4].

Zusammenfassung. Wasserlösliche Enzyme wurden mit saturierten alphatischen Kohlenwasserstoff-Diisozyanaten in Reaktion gebracht und die Bindung dieser Enzymderivate mit der Kohlenwasserstoffschicht stark gefördert. Bis zu 85% Katalysationstätigkeit der Schweinepankreas-Lipase konnte aus der Reaktionsmischung in Form einer beständigen Kohlenwasserstoff-Emulsion zurückgewonnen werden.

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