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PURIFICATION AND CHARACTERIZATION OF FULLY DEUTERATED ENZYMES*

S. ROKOP, L. GAJDA, S. PARMERTER, H. L. CRESPI AND J. J. KATZ
Chemistry Division, Argonne National Laboratory, Argonne, Ill. 60439 (U.S.A.)

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SUMMARY

Two fully deuterated enzymes, a bacterial alkaline phosphatase and a plant ribonuclease, have been isolated in a high degree of purity. Kinetic measurements and thermal studies indicate only slight differences in the tertiary structure of the fully deuterated enzymes as compared to the ordinary enzymes. However, the deuterated alkaline phosphatase shows a decreased rate of catalysis.

INTRODUCTION

Considerable literature exists on the effects of deuterium on enzymatic reactions[1]. Such studies have generally compared the action of ordinary enzymes on ^1H- and ^2H-substrates[2,3] or have examined the action of ordinary enzymes on hydrogen substrates in ^1H_2O and ^2H_2O media. Except for the hydrogen at exchangeable positions in the enzyme, the enzymes studied have had the usual isotopic composition. With the successful cultivation of fully deuterated organisms[4], however, it now becomes feasible to consider the preparation of fully deuterated enzymes and to begin the exploration of a new category of deuterium isotope effects of biological importance. A fully deuterated enzyme is defined as an enzyme containing deuterium in place of hydrogen at all non-exchangeable positions in the side chains of the constituent amino acids of the enzyme.

Side-chain deuteration of proteins has recently been shown to effect the stability and association of the algal chromoprotein phycocyanin[5]. The essentially complete substitution of deuterium for hydrogen at non-exchangeable carbon–hydrogen positions decreases the extent of subunit association[6]. These effects of side-chain deuteration suggested the desirability of extending such studies to fully deuterated enzymes, since effects of deuteration on living organisms may involve alterations in enzyme activity[7]. Some experiments with crude enzyme extracts prepared from deuterated organisms have been reported[8,9]. We, however, consider it imperative to work with highly purified enzymes because in the absence of accurate information about enzyme

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concentration no firm conclusions can be reached about the magnitude of deuterium isotope effects on the properties of a fully deuterated enzyme.

The experiments reported here have a direct bearing on the application of isotopic hybrid proteins to structure and enzyme studies. Methods have been developed[4,10,11] whereby otherwise fully deuterated proteins and enzymes can be selectively substituted (by biosynthesis) with amino acids containing ordinary ^1H in non-exchangeable positions. A deuterated protein into which particular ^1H-amino acids are substituted is termed an isotope hybrid protein. Isotope hybrid proteins are particularly useful for high resolution proton magnetic resonance studies. It is important to know, then, whether the information derived from the hybrid proteins is representative of the protein of ordinary isotopic composition. This paper presents comparative data obtained with preparations of two fully deuterated enzymes: (a) a bacterial alkaline phosphatase and (b) a plant ribonuclease. Comparison of the deuterated enzymes with the ordinary ^1H-enzymes supports the view that, although rates may be affected by isotopic substitution, little in the way of conformational differences between ordinary and fully deuterated proteins are observed. Proton magnetic resonance studies on isotope hybrid enzymes, in which the ^1H-containing amino acid side chains function as reporter groups, can thus be expected to provide valid information on enzyme function.

EXPERIMENTAL

Materials

DEAE- and CM-cellulose and *p*-nitrophenylphosphate were obtained from Mann Research Laboratories, yeast extract from Difco Laboratories, muramidase (lysozyme) and pancreatic ribonuclease from Worthington Biochemical Corp., and Tris from Fisher Scientific Co. Yeast nucleic acid was purified by the method of FRISCH-NIGGEMEYER AND REDDI[12]. *Escherichia coli* C-90, R^+, R_2^-, P^+ was a gift of Dr. Thomas Henderson, University of Arkansas Medical School and also of Dr. A. Garen, Yale University. Algal extract was prepared from fully deuterated *Scenedesmus obliquus* by the method of CRESPI *et al.*[13]. NH_4Cl and KH_2PO_4 were exchanged with ^2H_2O before use in deuterated media.

Ordinary and fully deuterated *Fremyella diplosiphon* (Indiana University Algae Culture Collection, No. 481), *Chlorella vulgaris* (397) and *Scenedesmus obliquus* (393) were cultivated by the methods of DABOLL *et al.*[14].

E. coli was grown in H_2O on modified salts of KORNBERG *et al.*[15] with 2% glucose and 0.1% yeast extract. In 99.7% ^2H_2O, 1% deuterated algae extract was substituted for the glucose and yeast extract. Bacteria were grown in 5-l batches at 27° with ample air and agitation. The pH was maintained near neutrality by periodic addition of base during the 2–3-day period of growth. Growth was followed with a Klett–Summerson colorimeter using a 420-mμ filter; harvest, by refrigerated centrifugation, was at 500–600 absorbance units.

Methods

Isolation of ribonuclease activity

The assay procedure was a modification of that of TUVE AND ANFINSEN[16].

Under our conditions, pH 6.8 for 30 min, one unit of activity corresponds to $9 \cdot 10^{-6}$ mg of pancreatic ribonuclease.

A mixture of 50 g of lyophilyzed algae and 800 ml of 0.04 M NaCl solution was stirred at room temperature for 30 min. Cell debris was removed by centrifugation at $1300 \times g$ for 30 min in an International Centrifuge, model V-2. The solid was again extracted for 30 min with 500 ml of 0.4 M NaCl solution.

The combined extracts were chilled to 5° and acidified to pH 2.0 by the slow addition of about 50 ml of 0.5 M H_2SO_4 with stirring. The mixture was chilled at 5° for 1 h and then centrifuged. This centrifugation was done at $1300 \times g$ for 30 min when working with deuterated algae but at $15\,000 \times g$ for 20 min when working with the ordinary algae, as the precipitate from the ordinary algae was quite gelatinous. The purple solid was discarded. The pH of the supernatant solution was adjusted to 5.0 by the addition of a few drops of concentrated NH_4OH and then the solution was dialyzed for 16 h against 3 l of distilled water at 3°. The mixture was centrifuged for 15 min at $15\,000 \times g$ to remove a small amount of solid.

The solution was allowed to pass through a CM-cellulose column, 1.7 cm × 9.0 cm, equilibrated with 0.01 M citrate buffer (pH 5.0). The flow rate was 20 ml/h. After a brief washing with citrate buffer, the column was washed with 0.05 M Tris–HCl (pH 7.6) until $A_{280\ m\mu}$ of the effluent was less than 0.05. About 200 ml of buffer were required. The column was then washed with Tris buffer made 0.1 M in NaCl until the $A_{280\ m\mu}$ of the effluent was less than 0.05. With a fraction collector, the enzyme was eluted with Tris buffer made 0.2 M in NaCl. The most active fractions were pooled and dialyzed.

In experiments with Chlorella and Scenedesmus cell rupture was achieved by passing a cold suspension of cells through a French press at 4000–5000 lb/inch^2.

Isolation of alkaline phosphatase

Alkaline phosphatase was extracted from *E. coli* cells using the technique of spheroplast formation described by MALMY AND HORECKER[17]. Spheroplasts were centrifuged at 4° in a Beckman-Spinco model L, rotor 30.2 at $20\,000 \times g$ (av.) for 15 min. Pellets were twice resuspended in buffer and centrifuged. All supernatant solutions were pooled. Purification of alkaline phosphatase was effected through a combination of the methods of MALMY AND HORECKER[17] and GAREN AND LEVINTHAL[18]. Except for growing the bacteria in ^2H_2O, subsequent purification was carried out in H_2O.

DEAE-cellulose was washed in 0.5 M NaOH, rinsed to neutrality with distilled water, washed in 0.5 M HCl, rinsed to neutrality and finally washed with the appropriate buffer. Columns of 1.7 mm × 6.0 mm and 2.5 mm × 10 mm were poured and then packed with 3 lb/inch^2 of air. The crude extract, in 33 mM Tris (pH 8.0) 20% sucrose, was fed slowly through a column previously equilibrated with 33 mM Tris (pH 8.0). After washing with 20–50 ml of 0.05 M NaCl, the enzyme was stripped from the column with 0.175 M NaCl. The eluate was collected in 3-ml fractions that were assayed for phosphatase activity and absorption at 278 mμ. Those fractions containing the bulk of the purified enzyme were combined for further purification.

A second DEAE-cellulose column was prepared with 0.01 M Tris (pH 8.0) containing 0.04 M NaCl. Purified enzyme from the first DEAE-cellulose column was adsorbed and then eluted with a linear NaCl gradient, 0.04–0.2 M buffered with 0.1 M Tris (pH 8.0). 3-ml fractions were collected and assayed as above. Tubes containing

pure alkaline phosphatase were combined and centrifuged to remove any traces of particulate matter. Enzyme solutions were stored at 4° for periods of months with no loss of activity.

The assay method was that of GAREN AND LEVINTHAL[18]. The formation of nitrophenol from *p*-nitrophenylphosphate was followed at 410 mμ with a Cary model 14 recording spectrophotometer. Routine assays were at room temperature. Thermostated cell holders were used in other experiments. The molar absorbance index at 278 mμ was taken to be 0.72 per mg[17,19]. Gel electrophoresis was performed in Tris–glycine buffer (pH 8.6) according to the methods outlined by ALLEN AND HYNCIK[20].

Michaelis constants were determined with Tris buffer (pH 8.0). Substrate concentrations varied from 1–0.01 mM; enzyme concentrations in reaction mixtures were 0.2 μg/ml or 0.15 μg/ml. Heat inactivation and reactivation experiments were conducted with solutions containing 0.02 mg/ml of enzyme that had been dialyzed against a solution containing 0.15 M Tris (pH 8.0), 0.01 M $MgCl_2$ and 15 mM NaCl. After heating, the solutions were quenched at 0° and immediately assayed. In experiments in which reactivation was followed, enzyme solutions were kept at 90° for 20 min, quenched in ice water and then warmed to 25° and assayed periodically.

RESULTS

Characterization of ribonuclease activity

The course of purification of the ordinary and deuterated ribonuclease activity is summarized in Tables I and II. Fig. 1 shows a representative elution pattern for the CM-cellulose column purification. Somewhat more activity was extracted from the deuterated algae, probably due to the greater fragility of the deuterated algae, which resulted in greater ease of extraction, rather than to a higher enzyme level. The final degree of purity was the same for both enzymes, and the maximum activity ever obtained upon a second chromatographic purification was 1900 units per ml per unit absorbance at 280 mμ.

TABLE I

DATA OF PURIFICATION OF RIBONUCLEASE FROM 25 g *F. diplosiphon* (H_2O)

Step	*Fraction*	*Volume (ml)*	*Ribonuclease (units/ml)*	$A_{280\ m\mu}$	*Units/ml per* $A_{280\ m\mu}$	*Total units*
1	Initial extract	575	6.3			3600
2	After acid treatment					
	Before dialysis	500	5.0	20.0	0.25	2500
	After dialysis	500	4.0	5.0	0.8	2000
3	Combined batch eluates from CM-cellulose					
	Before dialysis	91	19	0.88	22	1700
	After dialysis	91	18	0.61	29	1600
4	Combined fractions from column chromatography					
	Before dialysis	24	54	0.22	245	1300
	After dialysis	20	47	0.08	588	940

TABLE II

DATA OF PURIFICATION OF RIBONUCLEASE FROM 50 g *F. diplosiphon* (^2H_2O)

Step	*Fraction*	*Volume (ml)*	*Ribonuclease (units/ml)*	$A_{280\ m\mu}$	*Units/ml per* $A_{280\ m\mu}$	*Total units*
1	Initial extract	1170	9.6			11 200
2	After acid treatment					
	Before dialysis	1400	8.0			11 200
	After dialysis	1400	7.4			10 400
3	Combined batch eluated from CM-cellulose					
	Before dialysis	100	61			6 000
	After dialysis	105	67	0.78	86	7 000
4	Combined fractions from column chromatography					
	After dialysis	21.5	190	0.33	576	4 000
5	Combined fractions after rechromatography	20	126	0.12	1050	2 500

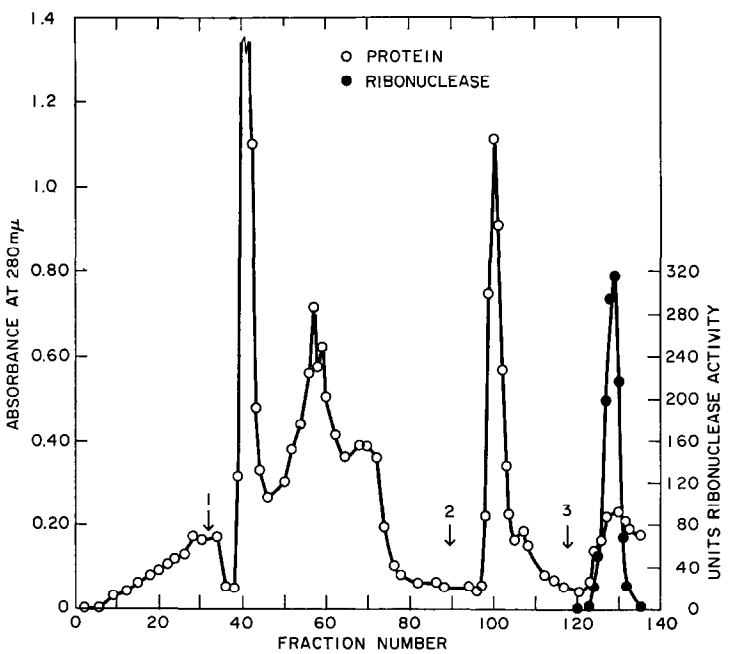


Fig. 1. Chromatography of extract from ordinary *F. diplosiphon* on CM-cellulose column. The column was loaded with crude extract after treatment with acid, neutralization to pH 5.0, and dialysis against distilled water. At Point 1, start 0.05 M Tris–HCl, pH 7.6. At Point 2, Tris buffer 0.1 M in NaCl. At Point 3, Tris buffer 0.2 M in NaCl. Fractions were 3.0 ml.

Maximal enzymatic activity for both the ordinary and deuterated enzyme was found in the pH range 5.5–7.0. The maximum for the deuterated enzyme appeared to have shifted the order of 0.2–0.3 pH units to lower pH. The pH optima are somewhat higher than those reported for the ribonuclease from spinach[16], corn[21] and tobacco leaves[12]. The enzyme attacked RNA core[22], indicating that it is similar to other plant ribonucleases in this respect. Under our conditions, both the ordinary and deuterated enzymes were very heat stable, and temperatures approaching 100° were necessary for inactivation. At 100°, a half-time of about 10 min was observed for the inactivation of both enzymes.

Small amounts of ribonuclease activity (of the order of 90 units per g of algae) were obtained from ruptured Chlorella cells, both ordinary and fully deuterated. Ruptured Scenedesmus, both ordinary and deuterated, showed no activity and a variety of other disruption and extraction procedures were uniformly unsuccessful in extracting any ribonuclease activity from Scenedesmus.

Characterization of alkaline phosphatases

The elution patterns from the second purification with DEAE-cellulose were similar for both the ordinary and deuterated enzymes. The first few fractions to show alkaline phosphatase activity often contained ultraviolet-absorbing impurities, especially in the case of the deuterated material. However, this impurity was minimized by pooling only middle fractions and, except for a few of our earliest preparations, the ultraviolet spectra of the final preparations of ordinary and fully deuterated enzymes were identical. It was also found helpful to purification to omit re-extraction of the deuterated spheroplasts. Polyacrylamide column gel electrophoresis of both enzymes showed but a single band when developed for protein or enzyme activity, and the chromatographic behavior of the deuterated alkaline phosphatase was indistinguishable from the ordinary enzyme. The specific activity of the ordinary enzyme was typically in the range of 2500–3000 units/h per mg, in agreement with other workers[17,19].

The pH optima of our preparations were determined with a series of Tris buffers ranging in pH from 7.20 to 8.95. The results of these experiments are shown in Fig. 2. The optimum pH is 8.4 for both the ordinary and deuterated enzymes.

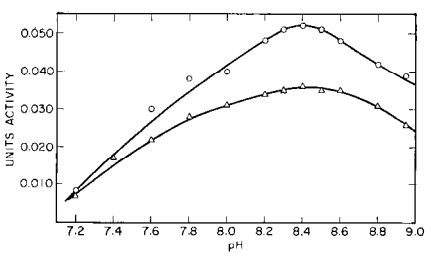


Fig. 2. The pH dependence of ordinary (○) and fully deuterated (△) alkaline phosphatase. These experiments were performed in a series of H_2O 1.0 M Tris buffers. All experiments were run at 25°.

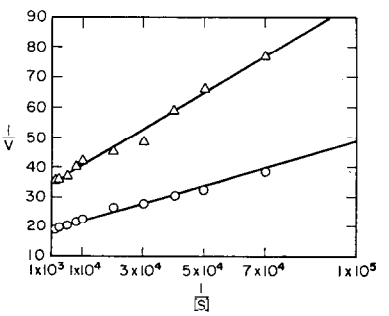
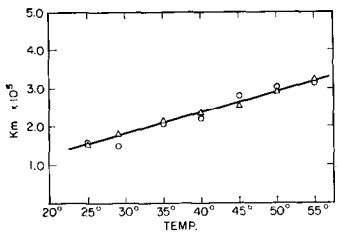


Fig. 3. Lineweaver–Burk plots obtained with ordinary (○) and fully deuterated (△) alkaline phosphatase at 25°, 1.0 M Tris. These data give Michaelis constants of $1.6 \cdot 10^{-5}$ M (ordinary) and $1.7 \cdot 10^{-5}$ M (deuterated).

Michaelis constants (K_m) were calculated from the usual Lineweaver–Burk plots, Fig. 3. At 25°, a value of $1.6 \cdot 10^{-5}$ M was obtained for ordinary alkaline phosphatase and $1.7 \cdot 10^{-5}$ M for the deuterated enzyme. Fig. 4 shows the Michaelis constant for the two enzymes as a function of temperature from 25 to 55°. The plot of K_m *versus* temperature is described by a single straight line of slope 0.05, so that at any particular temperature a single K_m value applies to both enzymes. However v_{max}



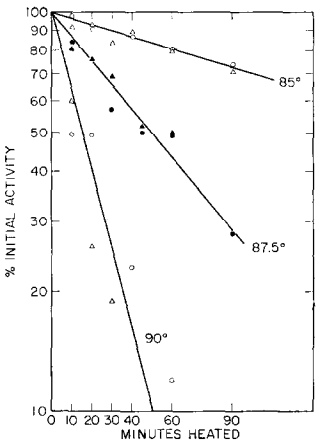


Fig. 4. The temperature dependence of the Michaelis constant for ordinary (○) and deuterated (△) alkaline phosphatase, 1.0 M Tris.

Fig. 5. Thermal inactivation of ordinary (○, ●, ○) and deuterated (△, ▲, △) alkaline phosphatase. There appears to be little or no effect of side-chain deuteration on the thermal inactivation of the enzyme.

for the deuterated enzyme appears to be considerably less than v_{max} for the ordinary enzyme. In 1.0 M Tris, as shown in Fig. 3, the reaction rate for the deuterated enzyme is decreased by a factor of 1.8, and in 0.05 M Tris or in 0.05 M Tris with 0.05 M NaCl, the reaction rate for deuterated alkaline phosphatase is down by the same factor of 1.8. This difference in v_{max} has been observed consistently throughout a number of separate enzyme preparations. The activation energies for the reaction with *p*-nitrophenylphosphate were found to be 5280 cal/mole for the ordinary enzyme and 5230 cal/mole for the deuterated enzyme. It is interesting to note that the same isotope effect is observed at both 1.0 and 0.05 M Tris, for at 0.05 M Tris only the hydrolytic reaction is observed while at 1.0 M Tris the sum of the hydrolytic and transfer (to Tris) reactions is measured. Reaction rates in 0.05 M Tris were depressed by about 20% as compared to 1.0 M Tris in agreement with the results of HEPPEL *et al.*[23].

Heat inactivation experiments were conducted at 90.0, 87.5, and 85.0°. In Fig. 5, the results are plotted as percent of initial activity as a function of time. Once again the two enzymes exhibited the same behavior. Reactivation experiments yielded results similar to those reported by HEPPEL *et al.*[23]. When the ordinary alkaline phosphatase was 50% inactivated, it recovered to the extent of 85% of its initial activity. When the deuterated enzyme was 50% inactivated, it recovered to the extent of 90% of its initial activity. If the enzymes were inactivated to the point where only about 20% of the initial activity remained, the ordinary enzyme recovered to about 45%

of its initial activity while the deuterated enzyme recovered to only 35% of its initial activity.

DISCUSSION

Previous work with ordinary and fully deuterated phycocyanins[24] indicates that, by the criterion of optical rotatory dispersion, side-chain deuteration can be expected to cause little or no change in the conformation of proteins. The results reported here are consistent with this view. The difference in v_{max} between the ordinary and deuterated enzymes may be explained in terms of a modification of the vibrational frequency distribution as discussed by PHILLIPSON[25]. In the deuterated enzyme, there would be less vibrational energy available at the active site and therefore a decreased reaction rate. The great similarity in the other measured properties, of the isotopically different enzymes also imply no serious conformational differences between the two.

The effects of temperature on the enzymes are consistent with previous results on phycocyanin. In the case of a phycocyanin that undergoes denaturation at 49°, side-chain deuteration lowers the denaturation temperature by about 7°. Phycocyanin from a thermophilic alga denatures at 60°, and deuteration lowers this temperature by 2°. Ordinary alkaline phosphatase is inactivated at 85–90° and the ribonuclease activity is stable to about 100°. We have observed no significant isotope effect on thermal denaturation of our deuterated enzymes. It thus appears that the higher the temperature of denaturation, the smaller is the effect of side-chain deuteration on the thermal stability of proteins. Our results also lend support to the suggestion that hydrophobic bonds are strongest in the region of 50° and become weaker at both higher and lower temperatures[26].

The finding that ribonuclease activity is essentially the same in both ordinary and deuterated algae indicates that the very high ribonucleic acid content of deuterated algae[27] is probably not due to the absence of this enzyme. In general, the findings reported here support the contention that deuterium may strongly affect the elaboration or control of enzymes rather than enzyme function *per se*[28]. Since large deuterium isotope effects have been reported in oxidation–reduction reactions[1], it may well be that the situation will be quite different when fully deuterated oxidation–reduction enzymes are studied.

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