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Liver Aldolase Anomeric Specificity†

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ABSTRACT: Stopped-flow kinetic studies of liver aldolase and of mixed liver–muscle aldolase catalyzed reactions of fructose 1,6-bisphosphate (FBP) have been carried out and interpreted by computer simulation. These experiments indicate no utilization or binding of the α anomer by the liver enzyme unlike the findings for either the muscle aldolase which binds the α anomer nonproductively or the yeast aldolase which catalyzes its cleavage. Both β-fructose 1,6-bisphosphate and its acyclic keto form may serve as substrates, necessitating the spontaneous anomerization of the α anomer before its utilization. Thus, liver aldolase cleaves 100% of the substrate present in the millisecond time scale because of the inability to bind α-FBP, allowing rapid spontaneous anomerization. This result fulfills earlier predictions of the differing specificities and substrate binding properties for aldolases from yeast, muscle, and liver.

Most enzymes using sugar phosphates have now been examined for their specificity toward the anomeric or acyclic forms of these substrates. Several interesting postulates concerning the role of the observed specificities in metabolic regulation have been put forward (Schray & Benkovic, 1978; Benkovic & Schray, 1976; Wurster & Hess, 1974; Koerner et al., 1977).

Muscle aldolase has been shown by Wurster & Hess (1973) as well as by Schray et al. (1975) to be specific for the β anomer of fructose 1,6-bisphosphate (FBP)[1] and to bind the

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α-FBP so tightly as to slow anomerization and inhibit its use as a substrate. In rapid kinetic experiments which employed mixtures of muscle aldolase and yeast apoaldolase, the latter authors have demonstrated the presence of an anomerase activity in the yeast enzyme which allows utilization of all substrate forms. Rose & O'Connell (1977) have confirmed use of the β anomer by muscle aldolase in continuous substrate generation experiments.

Previously we have offered a rationale for the differences in the class I muscle and class II yeast enzymes and predicted that the liver enzyme, though a class I enzyme closely related to muscle, should resemble the yeast enzyme in terms of its anomeric specificity and total substrate utilization (Schray & Benkovic, 1978; Benkovic & Schray, 1976). The results described in this paper show that while the anomeric specificity of the yeast and liver enzymes is different, the net substrate use is the same.

Materials and Methods

Liver aldolase was prepared from freshly excised rabbit livers according to the procedure of Chappel et al. (1976) with slight modification. The specific activity was 0.6 unit/mg, assayed according to Rutter et al. (1966). In this paper a unit is defined as 1 μmol of product formed per min under the conditions of our assay or that of the supplier. Care was taken to exclude sulfate from the kinetic experiments as this has been demonstrated to inhibit the coupling enzymes α-glycerophosphate dehydrogenase (EC 1.1.1.8) and triosephosphate isomerase (EC 5.3.1.1). Muscle aldolase (16 units/mg) and the coupling enzymes were obtained from Sigma Chemical Co. as lyophilized sulfate-free powders and were used without further purification. The rapid kinetic experiments were performed by using a Durrum Model D-134 stopped-flow instrument. Stopped-flow runs were made with muscle aldolase, liver aldolase, and muscle plus liver aldolase. In the muscle aldolase experiments, the final concentrations were 12 μM FBP, 20 units/mL muscle aldolase [23 μM sites calculated from (units/mol)(0.06 mg/unit) (4 sites/mol)(mol/160 000 mg)], 175 units/mL α-glycerophosphate dehydrogenase, 4320 units/mL triosephosphate isomerase, 30 μM NADH, 25 mM Tris-HCl, and 5 mM EDTA. The pH was 7.5 and the volume of the reaction mixture was ~0.6 mL. In the liver aldolase experiments, the conditions were the same as those in the muscle aldolase experiment except for the following: 4.5 units/mL liver aldolase (189 μM sites), 50 mM Tris-HCl, and 10 mM EDTA. In the mixed liver plus muscle aldolase experiments, the final concentrations were 12 μM FBP, 20 units/mL muscle aldolase (23 μM sites), 2.25 units/mL liver aldolase (93 μM sites), 175 units/mL α-glycerophosphate dehydrogenase, 4320 units/mL triosephosphate isomerase, 30 μM NADH, 50 mL Tris-HCl, and 10 mM EDTA. The pH and reaction volume were as described above.

The chemical reaction analysis modeling system (CRAMS) program, described previously by Fishbein et al. (1974), was utilized along with an IBM 370-168 computer to simulate the results of the stopped-flow experiment assuming various anomeric specificities. In this program, time–reaction progress curves are generated for given initial concentrations and given rate constants. The concentrations utilized for these simulations were those employed in the stopped-flow experiments. Michaelis constants were determined independently under the conditions of the stopped-flow experiments and assumed equal to true dissociation constants. It has been reported previously by Mehler (1963) and by Rose & O'Connell (1969) that monovalent anions affect the strength of the binding interactions and, hence, the necessity of determining the value of K_M under the experimental conditions employed.

[1] Abbreviations used: FBP, fructose 1,6-bisphosphate; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane.

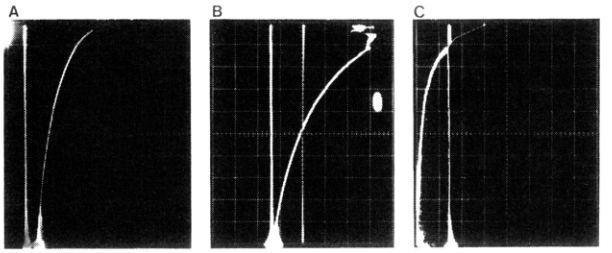


FIGURE 1: Stopped-flow oscilloscope traces of FBP cleavage by aldolase. (A) 20 units/mL (23.4 μM sites) muscle aldolase, 175 units/mL α-glycerophosphate dehydrogenase, 4320 units/mL triosephosphate isomerase, 30 μM NADH, 12 μM FBP, 25 mM Tris-HCl, and 5 mM EDTA, at pH 7.5. Slit width was 1 mm; vertical offset of 0.4 A_{340}; vertical scale of 0.5 A_{340}/division; horizontal scale of 0.1 s/division. For the infinite time trace, the vertical offset was 0.3 A_{340}. (B) 4.5 units/mL (187 μM sites) liver aldolase, 175 units/mL α-glycerophosphate dehydrogenase, 4320 units/mL triosephosphate isomerase, 30 μM NADH, 12 μM FBP, 50 mM Tris-HCl, and 10 mM EDTA, at pH 7.5. Slit width was 1 mm; vertical offset was 0.3 A_{340}; vertical scale of 0.5 A_{340}/division; horizontal scale of 0.2 s/division. (C) 2.25 units/mL (93 μM sites) liver aldolase, 20 units/mL (23.4 mM sites) muscle aldolase, 175 units/mL α-glycerophosphate dehydrogenase, 4320 units/mL triosephosphate isomerase, 30 μM NADH, 12 μM FBP, 50 mM Tris-HCl, and 10 mM EDTA, at pH 7.5. Slit width was 1 mm; vertical offset was 0.4 A_{340}; vertical scale of 0.05 A_{340}/division. Note: the vertical offset value is one grid division from the bottom of the grid. All runs were at 31 °C.

The dissociation constant for α-FBP to muscle aldolase was calculated from the observed α-FBP $\rightarrow$ β-FBP rate in the muscle aldolase, the known $\alpha \rightarrow \beta$ rate (Midelfort et al., 1976), and the known concentrations of enzyme and α-FBP. Thus, assuming that free α-FBP anomerizes at the normal rate and aldolase-bound α-FBP does not anomerize, $K_{diss} = 1.3 \times 10^{-6}$ M is obtained. The rate constants for the interconversions of the anomeric forms of FBP were those reported by Midelfort et al. (1976). The anomeric composition was assumed to be 4:1 β/α with less than 2% of the open-chain intermediate present in solution as previously reported (Gray, 1971; Midelfort et al., 1976). The overall reaction scheme utilized in the simulations is shown in Scheme I. By utilization of K_M values determined independently, the "on" step rate was assumed to be diffusion limited and the "off" step rate was then determined from the K_M. The usual value for a diffusion-controlled process of 10^8 M^{-1} s^{-1} was used. All concentrations were expressed in the program as micromolar concentrations and, therefore, this number becomes 10^2 μM^{-1} s^{-1}. In individual simulations the system was simplified by utilizing only the appropriate portion of this overall reaction scheme. The dissociation constants then used were adjusted appropriately; i.e., for the acyclic keto form only as substrate, the observed $K_M = 10^{-6}$ M for total substrate would be actually $K_M = 10^{-8}$ M for the carbonyl form which constitutes ~1% of substrate.

Results

The oscilloscope trace from a typical muscle aldolase stopped-flow experiment is shown in Figure 1A, and the first-order replot of the data is shown in Figure 2a. The curve in Figure 2a is clearly biphasic with the rapid initial phase ($k = 4.7$ s^{-1}) accounting for the utilization of ~74% of the initial substrate concentration and a slow phase ($k = 0.69$ s^{-1}) completing the reaction. These data are identical with those previously described (Schray et al., 1975; Wurster & Hess, 1974). The trace from a similar experiment employing liver

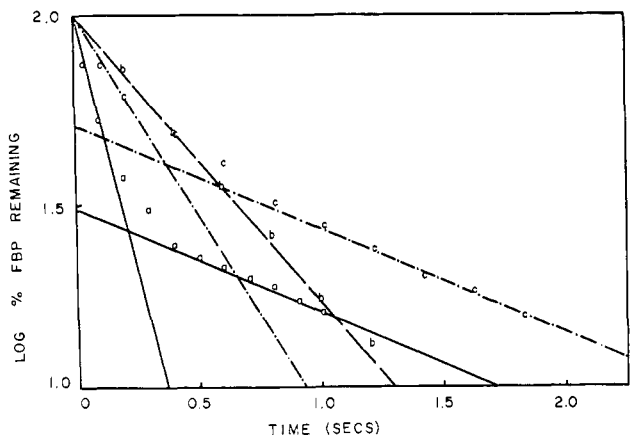


FIGURE 2: First-order replots for the experiments shown in Figure 1. (a) Muscle only (—); (b) liver only (– – –); (c) liver plus muscle (–·–).

aldolase is shown in Figure 1B, and the replot is shown in Figure 2b. Unlike the muscle aldolase experiment, this replot is linear with $k = 1.7\ s^{-1}$ which is in reasonable agreement with the turnover number under dilute enzyme conditions ($1.8\ s^{-1}$ at 25 °C) (Rutter, 1964).

Though these data clearly demonstrate that muscle and liver aldolases interact differently with the anomeric substrate forms, they do not define that difference. In Figure 1C is presented the trace from a typical stopped-flow experiment with both muscle aldolase and liver aldolase present. The replot shown in Figure 2c is biphasic with the initial phase ($k = 2.5\ s^{-1}$) accounting for ~50% of the initial substrate concentration, followed by a slow second phase ($k = 0.69\ s^{-1}$).

The reduction from 74% utilization seen with the muscle aldolase to the 50% utilization with the liver and mixed aldolase systems may arise in part from the technical difficulties inherent in mixing solutions which have a high protein concentration. As a result it was not possible to obtain reliable intercept data either from the first 100 ms of the liver aldolase stopped-flow experiment or from the liver aldolase plus muscle aldolase stopped-flow experiment. Thus, $t = 100$ ms was arbitrarily set to $t = 0$ in the replot. However, as seen in latter simulations, competition for the α-FBP substrate form by aldolase enzymes of differing k_{cat} also produces a change in the intercept and a change in the apparent first-phase rate. Nevertheless, the second-phase rate is the same in both the muscle and the mixed aldolase experiments and corresponds to the muscle-inhibited α-FBP anomerization as discussed previously (Schray et al., 1975; Midelfort et al., 1976).

Since reactions with multisubstrate forms and mixed enzymes are complex, a minimal scheme was devised and computer simulations of the various substrate utilization models were run. The complete Scheme I is shown, and appropriate rate constants were set to zero to create the desired model (see Materials and Methods for further details).

Competitive binding simulation between the two Michaelis complexes (liver aldolase–FBP and muscle aldolase–FBP) in the absence of product-forming steps [utilizing the ICRS program of Shiendell & Magagnose (1976) and a Modcomp II/III] reveals virtually complete substrate binding (93%) at 1 ms. Distribution between the liver and muscle enzymes depends on the assumptions made but generally favors the liver enzyme complex; for example, the values shown in the computer scheme yield a distribution of ~75% substrate bound to liver enzyme and 15% bound to muscle enzyme.

The results of a computer simulation of only α-anomer utilization by liver aldolase with no β-anomer or acyclic keto

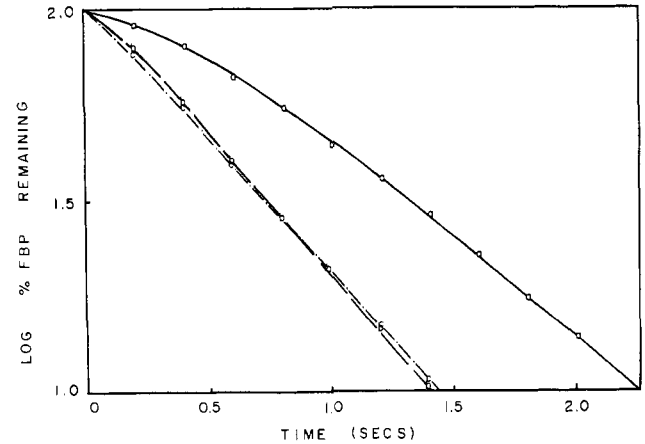


FIGURE 3: Simulations of utilization of different substrate forms by liver aldolase. (a) Use of α-FBP only with no binding of other forms (—); (b) use of open chain FBP with no binding of other forms (– – –); (c) use of β-FBP only with no binding of other forms (–·–).

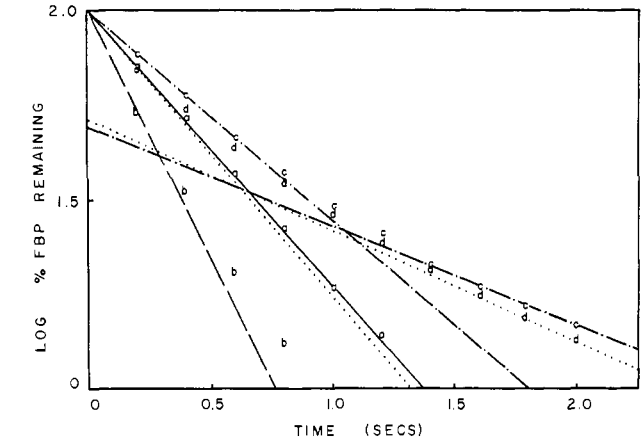


FIGURE 4: Simulations of utilization of different substrate forms by liver aldolase in the mixed liver aldolase plus muscle aldolase experiment. (a) Use of α- and β-FBP (—); (b) use of α-FBP with no binding of other substrate forms (– – –); (c) use of β-FBP with no binding of other substrate forms (–·–); (d) use of open chain FBP with no binding of other substrate forms (····).

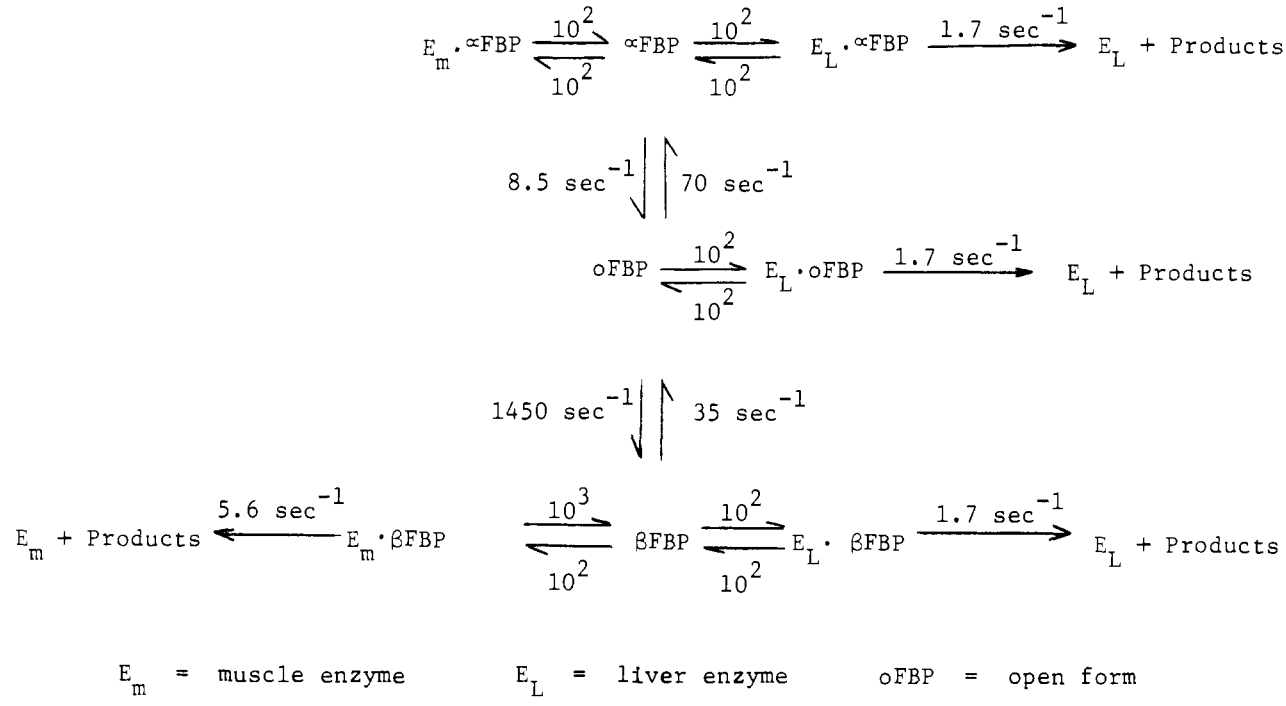
binding are shown in Figure 3a as a plot of log % FBP remaining vs. time. Under the conditions of these experiments, it should be remembered that all α anomer present initially would be bound and cleaved in the first turnover. The progress curve consists of a long lag period (~400 ms), followed by a first-order utilization of the FBP. This results from the accumulation of the enzyme–α-FBP complex following $\beta \rightarrow \alpha$ anomerization (cleavage being slower than $\beta \rightarrow \alpha$ anomerization). The cleavage rate, of course, increases as the enzyme–α-FBP complex concentration rises.

A similar plot simulating the exclusive utilization of the acyclic keto form of FBP is shown in Figure 3b. This plot has a shorter lag period (~80 ms), followed by a first-order disappearance of FBP. The lag phase is shorter than in the previous case since $\beta \rightarrow$ keto-FBP is more rapid than $\beta \rightarrow \alpha$ so that the enzyme–keto-FBP concentration is maximized more rapidly.

In Figure 3c is shown the plot simulating liver aldolase utilization of only the β anomer of FBP. This plot is first order for greater than three half-lives of FBP consumption since the decay of the enzyme–β-FBP complex dominates at least 80% of the reaction.

The simulated first-order plots for the mixture of muscle plus liver aldolase under the same conditions as the stopped-flow experiment are shown in Figure 4a–d. In all cases the

Scheme I



muscle enzyme utilizes β-FBP and binds α-FBP very tightly as shown previously. In Figure 4a use of both the α- and β-FBP by the liver enzyme is assumed. The plot is linear for greater than three half-lives of FBP disappearance as all of the substrate is undergoing first-order decay from the enzyme–substrate complexes.

A similar explanation also applies to the simulation depicted in Figure 4b when employment of only the α anomer of FBP by the liver enzyme is assumed. The β anomer can be cleaved by the muscle enzyme in a first-order decay process while all α-FBP is lost through the liver enzyme–α-FBP complex. This is more complex first-order decay supplemented by transfer of α from muscle to liver enzyme (loss of α from muscle enzyme is more rapid than cleavage by liver enzyme). Thus, this simulation also gives a nearly linear plot for three half-lives of FBP utilization. When the simulation is performed assuming utilization of only the β anomer of FBP by the liver enzyme, the progress curve obtained is that shown in Figure 4c. Finally, the result of a similar simulation assuming that liver aldolase uses only the open form of FBP is shown in Figure 4d. Note that these curves are biphasic due to the inability of either enzyme to use α-FBP directly and the fact that cleavage of β-FBP is more rapid than release of α-FBP from muscle, followed by anomerization or ring opening. Thus, a distinct phase due to α-FBP use is seen.

Obviously, the slopes and intercepts may be varied by altering the constants used in the simulation, but an arbitrary fine tuning of constants to match the simulation perfectly to the experiment is unwarranted in that virtually all the constants may reasonably be altered to some extent. Thus, there is no unique set which best fits the data.

Discussion

We have previously shown (Schray et al., 1975) that aldolases from muscle and yeast differ in the ability to cleave the anomeric forms of FBP. The muscle enzyme is well designed for use of β-FBP but not of α-FBP. The yeast enzyme is well suited for access into both the α- and β-FBP pools. We have postulated (Schray & Benkovic, 1978; Benkovic & Schray, 1976) that this difference is related to metabolic roles of the enzymes. Muscle aldolase is suited for efficient glycolysis by using as substrate the product of phosphofructokinase as this is the most important function for muscle tissue. The yeast enzyme is efficient in both glycolytic and gluconeogenic directions, being anomerically coupled to both phosphofructokinase and fructose bisphosphatase in keeping with the requirements of the yeast cell for capability in both directions. An alternate explanation for the observed differences is, however, to ascribe them to the fact that muscle contains a class I and yeast contains a class II aldolase. Liver, like muscle, of course, has a class I aldolase, but like yeast it must have efficient gluconeogenic capability and thus is an excellent case to test the above postulation.

As elucidated previously, the biphasic curve substrate utilization by muscle aldolase is due to use of the β anomer (and the free carbonyl) as the substrate with tight binding of the α anomer ($K_{diss} = 1.3 \times 10^{-6}$ M as calculated under Materials and Methods) which slows the net anomerization of $\alpha \rightarrow \beta$ to a rate observable on this time scale. The observation of first-order decomposition of 100% of substrate by liver aldolase demonstrates that liver interacts with the substrate forms differently than muscle and with a net result the same as that of yeast aldolase which uses both substrate forms by catalyzing their anomerization. Thus, metabolically the liver enzyme is similar to yeast in having rapid access to the total FBP pool so that the above postulate is supported.

As can be seen from the computer simulations, this first-order substrate use does not define the substrate forms used by liver aldolase, however. Only the case of α-FBP specificity may be eliminated by failure to see the predicted slow lag. Use of the keto form only, whose short lag would be undetected in these experiments, the β form only, or α and β through their nonenzyme conversion to the keto is compatible with the experimental result.

The mixed aldolase experiment yields, however, additional information. As can be seen from the simulation, the α- and β-FBP use by liver does not produce a biphasic plot and can thus be ruled out. Likewise, α-FBP employment only is

confirmed as incorrect. Specificity toward β-FBP only, the keto form only, or both remains a viable explanation. We presently disfavor the sole use of the acyclic species because of (1) cyclic analogue binding evidence which will be presented in a subsequent paper and (2) the simple consideration that the K_M values of $\sim 10^{-6}$ M (Mehler, 1963; Rose & O'Connel, 1969) for total substrate would require an unreasonably low $K_M \simeq 10^{-8}$ M for the keto form.

These experiments rule out an anomerase activity for the liver enzyme in that neither α-FBP alone nor in conjunction with β-FBP is used as a substrate and no catalysis of muscle aldolase use of α-FBP by liver aldolase is seen as with yeast aldolase. Thus, in evolutionary terms, it would appear that the muscle enzyme may have been modified to form the liver enzyme which functions well in both directions as does the yeast. However, this was not done by employing the α form as a substrate or achieving an anomerase activity but simply by altering the active site so that the α anomer is not bound. Consequently, a rapid spontaneous anomerization proceeds to maintain α-FBP ⇄ β-FBP equilibrium. It remains to be seen whether this spontaneous rate is rapid enough or whether a separate anomerase activity is required. Interestingly, 2-keto-3-deoxygluconate-6-P aldolase has been shown to use the free carbonyl form of its substrate, thus allowing it to be coupled to the preceding enzyme gluconate-6-P dehydratase which produces an acyclic form of the substrate, the enol (Midelfort et al., 1977).

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A Structural Study of the Hydrophobic Box Region of Lysozyme in Solution Using Nuclear Overhauser Effects†

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ABSTRACT: Saturation of specific proton nuclear magnetic resonance (NMR) signals from residues in the hydrophobic box region of lysozyme (EC 3.2.1.17) has enabled negative nuclear Overhauser effects to be measured on the resonances of nearby protons. The assignments of resonances reported previously have been examined, and most have been confirmed. In conjunction with spin-decoupling methods, new assignments could be made so that assignments for some 70 resonances of 25 residues in lysozyme are now known. A high correlation was observed between the observed nuclear Overhauser effects and interproton distances calculated from crystallographic data. This indicates that the average structure of this region of lysozyme in the crystalline state is maintained in solution, that substantial populations of structures very different from this do not exist, and that the nuclear Overhauser technique can be applied in a straightforward manner to obtain structural data in solution at the 1-Å level.

A full understanding of the folding, conformation, and function of a protein requires that the structure and dynamics of individual groups of the molecule in solution are defined and that any changes in these caused, for example, by substrate binding or partial unfolding can be described. The information about protein structure in the crystalline state is essentially static and must be supplemented by information from other techniques. For this reason, ^{1}H NMR studies of an enzyme, lysozyme, in solution are being performed (McDonald & Phillips, 1970; Campbell et al., 1975a; Dobson, 1977).

Although the NMR studies of lysozyme have revealed novel information about the protein structure, dynamics, and conformational changes, the information obtained has not yet allowed local structure and dynamics in solution to be defined with great accuracy. The problem is that the large number of nuclei in a protein results in the NMR parameters for each nucleus being determined by the sum of a large number of

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