essentially completely masks the influence of bound NADH. These findings support the idea that the sugar moiety of a UDP-sugar intervenes spatially between the nicotinamide coenzyme and the catalytic site thiol.

In summary, UDPGDH bears many of the marks of the half-of-the-sites enzymes and may operate catalytically by a flip-flop mechanism as recently suggested (Ordman and Kirkwood, 1976). It is clear that the catalytic site thiol groups interact with each other when alkylated. Events at one site are perceived by another site, this being a basic ingredient of any flip-flop mechanisms.

Acknowledgment

The technical assistance of Marie Bowser is gratefully acknowledged for certain aspects of the research reported here.

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Nuclear Magnetic Resonance Studies of D_2O–Substrate Exchange Reactions Catalyzed by Glutamic Pyruvic and Glutamic Oxaloacetic Transaminases†

U. M. Babu and R. B. Johnston*

ABSTRACT: Nuclear magnetic resonance studies in D_2O (>90%) with glutamic pyruvate transaminase (GTP) (2.6.1.2) demonstrate that this enzyme catalyzes the rapid exchange of both the α and β hydrogens of L-alanine, the exchange of only one α hydrogen of glycine, and the β hydrogens of pyruvate and fluoropyruvate. When the β hydrogens of L-alanine undergo the enzyme-catalyzed exchange, the product may have 1, 2, or 3 of β hydrogens exchanged. The exchange is stimulated by the addition of catalytic amounts of copartner of transaminations reaction. A mechanism is proposed for an extention of the conjugated system to include the α and β carbons to explain the labilization of the β hydrogens.

The most widely accepted mechanism of action for enzymatic transamination was proposed independently by Braunstein and Snell (Braunstein and Shemyakin, 1953; Metzler et al., 1954). This mechanism which was based prinicipally on extensive studies with model compounds involves the formation of the Schiff's base which results in a labilization of the α hydrogen of the amino acid. Early experiments by Grisolia and Burris (1954) and by Hilton et al. (1954) demonstrated that GOT[1] catalyzes the α-hydrogen exchange of glutamate. The first suggestion that the enzyme mechanism may be more complicated in that an interaction may also occur at the hydrogens of the β carbon was the observation of Oshima and Tamiya (1959, 1961) who found that the infrared spectra of L-alanine isolated after ion-exchange chromatography after it had been

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[1] Abbreviations used: NMR, nuclear magnetic resonance; GPT and GOT, glutamic pyruvic and glutamic oxaloacetic transaminases.

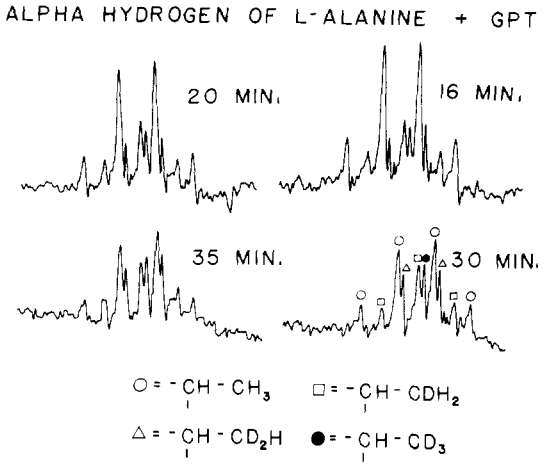

FIGURE 1: NMR spectra of the α hydrogen of L-alanine with GPT. The reaction mixtures contained 100 nmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 0.5 μmol of pyruvate, 9.3 units of GPT, and 100 μmol of L-alanine in a total volume of 0.46 ml. The spectra were taken on a deuterium-decoupled Varian XL-100 NMR spectrometer.

incubated with α-ketoglutarate and relatively large amounts of an enzyme fraction containing GPT activity in D_2O showed the exchange of both the α and the β hydrogens of L-alanine. The interpretation of their results has been questioned by Harley-Mason (1961) who suggested that the β exchange may be due to a tautomerization of pyruvate or a pyridoxamine–pyruvate complex. The possibility that this might be the case could not be excluded by the experimental technique of Oshima and Tamiya since, during the time required for reisolation, considerable nonenzymatic exchange of pyruvate occurs. Golichowski et al. (1971), using NMR techniques demonstrated that GPT catalyzes a rapid exchange of the β hydrogens of alanine. Further studies of the exchange catalysis by the transaminases were conducted in order to compare the exchange properties of the closely related pyridoxal phosphate requiring alanine racemase (Babu and Johnston, 1974). The experiments reported here show perhaps more convincingly by an independent method that the purified preparations of GPT do indeed catalyze an exchange of β hydrogens of L-alanine and the β hydrogens of pyruvate with D_2O. These exchanges are enzyme catalyzed and are dependent on the presence of catalytic amounts of corresponding copartner of the transamination reaction (Babu and Johnston, 1974).

Our experiments when carried out with glycine in the place of L-alanine confirm the observations of Besmer and Arigoni (1968) that only one hydrogen of glycine was exchanged with D_2O. Whelan and Long (1969) have reported that GOT catalyzes the exchange of the α and β hydrogens of glutamate but GPT catalyzes only the α-hydrogen exchange. We have found that glutamic oxaloacetic transaminase (GOT) also catalyzes the exchange of both the α and the β hydrogens of L-alanine but at a much slower rate than GPT. With both enzymes the rates of exchange of both the α and the β hydrogens of L-alanine with D_2O are the same order of magnitude. In addition to pyruvate, GPT also catalyzes the exchange with D_2O of the β hydrogens of fluoropyruvate and both the α an the β hydrogens of L-aspartate. GOT catalyzes the exchange of both the α and the β hydrogens of L-asparate with D_2O. With L-aspartate the rate of exchange of α hydrogen is much faster than that of β hydrogens. Recently, Walter et al. (1975) and Cooper (1976) have confirmed our findings of the GPT-catalyzed β-hydrogen exchange of alanine. In this paper is presented experimental evidence for (1) the GPT-catalyzed exchange of the hydrogens of pyruvate, (2) the stimulation of the exchange reaction by copartners of the transaminase system, and (3) the ability of the enzyme–substrate complex to exchange 1, 2, or 3 β hydrogens. A preliminary report of this work has appeared (Babu et al., 1974).

Experimental Section

Materials. Three different preparations of GPT were obtained from Sigma Chemical Co. One of these of specific activity 93 μmol min^{-1} mg^{-1} was prepared for Sigma and the other two preparations were prepared by Sigma as an ammonium sulfate suspension of specific activity 110 μmol min^{-1} mg^{-1} or lyophilized powder of specific activity 125 μmol min^{-1} mg^{-1} protein. These preparations gave similar results. GOT of specific activity 230 μmol min^{-1} mg^{-1} protein was also purchased from Sigma as an ammonium sulfate suspension. D_2O (99.8%) was obtained from NMR specialties. Sigma was also the source for β-alanine, glycine, L-glutamate, α-ketoglutarate, pyruvate, fluoropyruvate, taurine, and L-aspartate. Matheson Coleman and Bell supplied isopropylamine. L-Valine and L-serine were purchased from General Biochemicals. Mann Research Laboratories provided DL-alanine. Calbiochem supplied 1-aminoethylphosphonic acid. β-Chloro-DL-alanine was obtained as a hydrochloride from Cyclo Chemical. The other materials were the same as previously described (Diven et al., 1964; Johnston et al., 1966).

Methods. The exchange reaction was conducted in a NMR tube containing 100 nmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 100 μmol of the substrate, and 2.0 μmol of α-ketoglutarate or pyruvate in 0.45 ml of D_2O and 9.3 units (μmol/min) of GPT (or 18.0 units (μmol/min) of GOT) in 10 μl of water. The substrate solution was added to initiate the reaction which was carried out at 37 °C. At appropriate time intervals, the NMR spectra were taken on a deuterium-decoupled Varian XL-100 NMR spectrometer maintained at 37 °C. The area under the curves was determined by planimeter to calculate the extent of exchange.

Results

Exchange Studies with L-Alanine. Figure 1 shows the composite spectra of the α hydrogen of L-alanine taken on a deuterium-decoupled Varian XL-100 NMR spectrometer at various time intervals of the products which result from the enzyme-catalyzed exchange with D_2O. The assignment of each peak to a specific structure is indicated on the 30-min spectrum. The α-CH spectra, although it is diminishing throughout the course of the reaction due to the formation of -CD, clearly show the presence of the intermediates such as $-CHCDH_2$, $-CHCD_2H$, and $-CHCD_3$ in addition to $-CHCH_3$ of alanine. The spectra of the α hydrogen through coupling with the β hydrogens reflect the exchanges occurring at the β carbon. The β hydrogens thus show all possible exchanges with D_2O without the exchange of α hydrogen with D_2O. The appearance of a relatively large proportion of $-CHCD_3$ at the 16-min spectrum suggests that as many as all the three β hydrogens of L-alanine can exchange in its interaction with the enzyme. Thus the $-CHCD_3$ does not necessarily arise by the successive formation and dissociation of enzyme–substrate complexes in which first the monodeuterio- and then the dideuteriomethyl derivatives would have to exist in solution as obligatory intermediates.

Transaminase-catalyzed exchange of the α hydrogen of L-alanine can be easily followed by its coupling to the β hy-

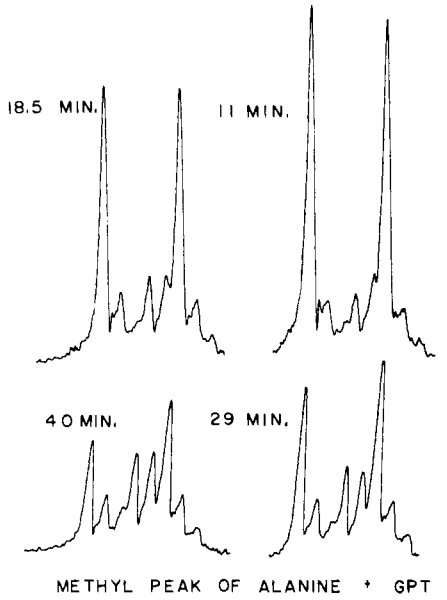

FIGURE 2: NMR spectra of the β hydrogens of L-alanine with GPT. Same experiment as in Figure 1.

drogens. The spectra of the β hydrogens of L-alanine in the same experiment as in Figure 1 are shown in Figure 2. The two large peaks in the 11-min spectrum correspond to the unexchanged $-CHCH_3$ of L-alanine. Throughout the time course of the reaction, the disappearance of this doublet can be due to (1) the exchange of the β hydrogens with D_2O or (2) the exchange of the α hydrogen with D_2O which would then cause the doublet to collapse into a large singlet centered between the doublet as observed in the case of alanine racemase catalyzed exchange (Babu and Johnston, 1974). The disappearance of the doublet without the corresponding increase of any of the other peaks including the singlet shows that the β hydrogens are exchanging at a rate as fast as or faster than the α hydrogen of L-alanine with D_2O. The occurrence of a number of peaks at longer time intervals indicates that the several combinations of α and β exchanges which are shown in Figure 1 exist. The small but significant peak centered between the doublet corresponds to $-CDCH_3$ of alanine. The existence of this peak indicates that α hydrogen can exchange independent of β-hydrogen exchange but the number of such exchanges is relatively small. GPT thus catalyzed a rapid exchange of both the α and β hydrogens of L-alanine with D_2O. All possible exchanges are observed including a small proportion of the exchanged products in which the exchanges of α and β hydrogens have occurred independently of each other.

The Effect of Keto Acids on Alanine Exchange. The exchange of the α and the β hydrogens of L-alanine with the medium as catalyzed by GPT is greatly accelerated by the addition of catalytic amounts of pyruvate or α-ketoglutarate. The effect of keto acids on the exchange of α and β hydrogens of L-alanine is shown in Table I. In the absence of any added keto acid, the exchange is not complete even after 10 h of incubation with the enzyme at 37 °C. The addition of 0.1 μmol of pyruvate increases the rate of exchange by five to eight times. Further addition of pyruvate up to 5.0 μmol increases the rate of exchange but in a nonlinear fashion. A similar stimulation is observed by the addition of α-ketoglutarate. However, in the presence of L-glutamate the stimulation of D_2O–L-alanine exchange by α-ketoglutarate shows a considerable time lag. This suggests that the stimulation by α-ketoglutarate is due to the formation of pyruvate as a result of transamination and it is the pyruvate which is causing the stimulation of the D_2O–L-alanine exchange. This is further suggested by the observation that L-glutamate inhibits the stimulation by α-ketoglutarate but not pyruvate stimulation of L-alanine exchange. Fluoropyruvate also stimulates the α- and the β-hydrogen exchange but not as rapidly as pyruvate. L-Alanine and L-glutamate do not accelerate the D_2O–substrate exchange of each other. The copartner of the coenzyme (in the case pyridoxamine phosphate) does not stimulate the exchange of alanine. Only keto acids which interact with the enzyme can stimulate the D_2O–L-alanine exchange.

TABLE I: Stimulation of the GPT-Catalyzed Exchange of the α and β Hydrogens of Alanine by Copartners and Analogues of Copartners of the Transamination Reaction.[a]

Description	Exchange Rate k_{1st} Order ($s^{-1} \times 10^{-5}$)
No addition	2.7 ± 0.4
+ pyruvate (0.1 μmol)	25 ± 3
+ pyruvate (1.0 μmol)	32 ± 3
+ pyruvate (2.0 μmol)	53 ± 3
+ pyruvate (5.0 μmol)	76 ± 1
+ α-ketoglutarate (2.0 μmol)	51 ± 1
+ α-ketoglutarate (5.0 μmol)	69 ± 3
+ α-ketoglutarate (2.0 μmol) and L-glutamate (6.0 μmol)	10 ± 2
+ pyruvate (1.0 μmol) and L-glutamate (6.0 μmol)	32 ± 1
+ L-glutamate (6.0 μmol)	2.9 ± 0.3
+ fluoropyruvate (5.0 μmol)	10 ± 2
+ pyridoxamine phosphate (2.0 μmol)	2.5 ± 0.6
+ β-chloro-DL-alanine (10 μmol)	0

[a] The reaction mixture contained 0.1 μmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 9.3 units of GPT, and 100 μmol of L-alanine in a total volume of 0.46 ml D_2O. Measurements were made at 2, 10, 20, 30, 40, 120, and 600 min. First-order rate constants were calculated from the appropriate time intervals.

GPT Catalyzed Exchange of Pyruvate. The exchange of the β hydrogens of pyruvate catalyzed by GPT is shown in Figure 3. The area under the curve B corresponds to 54% exchange in 2 h of incubation at 37 °C. After 6 h complete exchange occurs in the presence of the enzyme (curve D), while in the absence of the enzyme after this time interval (curve F) no more than 10% exchange can be observed. After longer periods, 27.25 h, complete nonenzymatic exchange was observed (curve G). The nonenzymatic exchange is too slow to account for the rapid enzymatic exchange observed with L-alanine and pyruvate. Since the nonenzymatic blank contained the same amount of added pyridoxamine phosphate as the enzymatic mixture, the formation of the Schiff base is not by itself sufficient to explain the enzymatic exchange, although the exchange observed nonenzymatically is probably due to the presence of pyridoxamine phosphate since pyridoxamine stimulates the rate of nonenzymic pyruvate exchange (Table III).

Effect of Amino Acids on Pyruvate Exchange. The rate of enzymatic exchange of the β hydrogens of pyruvate with D_2O is stimulated by catalytic amounts of amino acids as shown in Table II. The addition of up to 10 μmol of L-alanine stimulates the β-hydrogen exchange of pyruvate in a nonlinear fashion. The stimulation of pyruvate exchange by L-alanine is not as great as the stimulation of L-alanine exchange by pyruvate.

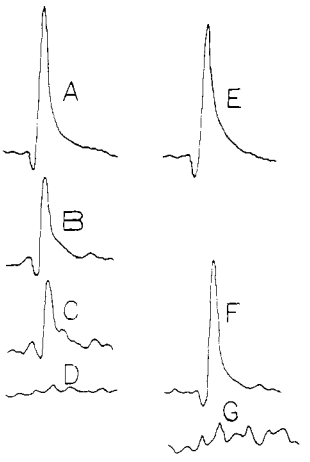

FIGURE 3: NMR spectra of the methyl peak of pyruvate with GPT. The reaction mixture contained 100 nmol of pyridoxamine phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 9.3 units of GPT, and 100 μmol of sodium pyruvate in a total volume of 0.46 ml. The spectra were taken on a Varian A-60 NMR spectrometer. A represents the spectrum at zero time, B at 2 h, C at 4.25 h, and D at 6 h. E, F, and G represent the spectra of pyruvate without any GPT at 0 time, 6 h, and 27.25 h, respectively.

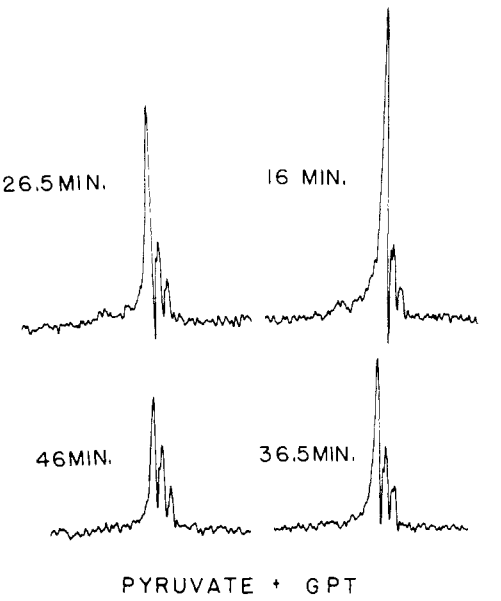

FIGURE 4: NMR spectra of methyl peak of pyruvate with GPT. The reaction mixture was the same as in Figure 3, except 5.0 μmol of L-alanine was added. The spectra were taken on a deuterium-decoupled Varian XL-100 NMR spectrometer.

TABLE II: Effect of Catalytic Amounts of Amino Acids on the GPT-Catalyzed Exchange of β Hydrogens of Pyruvate.[a]

Description	Exchange Rate K_{1st} Order ($s^{-1} \times 10^{-5}$)
No addition	4.3 ± 0.4
− enzyme	0.23 ± 0.02
+ L-alanine (1.0 μmol)	9.4 ± 0.7
+ L-alanine (5.0 μmol)	15 ± 1
+ L-alanine (10.0 μmol)	35 ± 8
+ α-ketoglutarate (5.0 μmol)	2.7 ± 0.2
+ L-glutamate (10 μmol)	4.5 ± 0.3
+ isopropylamine (10.0 μmol)	4.1 ± 0.1
+ glycine (10.0 μmol)	3.6 ± 0.9

[a] The reaction mixture contained 0.1 μmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 9.3 units of GPT, and 100 μmol of pyruvate in a total volume of 0.46 ml of D_2O. Measurements were made at 10, 30, 60, 90, 120, and 360 min. First-order rate constants were calculated from the appropriate time intervals.

TABLE III: The Effect of Pyridoxamine on the Nonenzymatic Exchange of the β Hydrogens of Pyruvate.[a]

Description	μmol
No addition	10
+ 0.1 μmol of pyridoxamine phosphate	15
+ 0.1 μmol of pyridoxamine phosphate	25
+ 10 μmol of pyridoxamine phosphate	85

[a] The reaction mixture contained 100 μmol of potassium phosphate buffer (pD 7.9) and 100 μmol of sodium pyruvate in a total volume of 0.46 ml of D_2O. Incubation time was 120 min.

This may reflect the higher affinity of the enzyme for the keto acid (Saier and Jenkins, 1967). In the absence of the copartner of the transamination reaction the rate of enzymatic exchange of L-alanine is similar to that of pyruvate exchange. This may be due to the formation of the copartner of the transamination by transamination with the coenzyme. In the presence of added copartner the rate of exchange of L-alanine is much faster than that of pyruvate. Thus under conditions where both the partners are present, the rate of exchange with L-alanine cannot be due to an exchange of pyruvate followed by transamination. Neither α-ketoglutarate nor isopropylamine has any stimulatory effect on the pyruvate exchange. L-Glutamate stimulates pyruvate exchange but not as greatly as L-alanine. Thus the transaminase-catalyzed exchange of β hydrogens of pyruvate is stimulated by the amino acids that react in the transaminase reaction and the copartner is the most effective.

The spectra of the GPT-catalyzed exchange of pyruvate as a function of time as recorded on a deuterium-decoupled Varian XL-100 NMR spectrometer are shown in Figure 4. The resolution obtained with this instrument by means of deuterium decoupling shows peaks corresponding to CH_3- (the largest peak), CH_2D- and CHD_2- of pyruvate. These exchanges correspond to those observed with the β hydrogens of L-alanine. The ratio of the latter two peaks remains fairly constant, indicating a very early appearance of dideuteriopyruvate. Thus this exchange, like the L-alanine exchange, does not necessarily occur by the formation and dissociation of a series enzyme-substrate complexes each of which exchanges one hydrogen.

Effect of pD on the Enzymatic Exchange of L-Alanine. The pD profile of the enzymatic exchange activity is given in Figure 5. There is a pD optimum around 8.1, and above 8.1 the exchange activity declines. The general shape of the curve and the position of the optimum corresponds closely to the pH-activity curve for transamination reported by Jenkins (Saier and Jenkins, 1967). There was no nonenzymatic exchange of L-alanine with D_2O at any of the pD values tested. The exchange of the β hydrogens was as fast as or faster than the exchange of the α hydrogens of L-alanine at all the pD's tested. The decreased rate of exchange at pD values greater than 8.1 is of special significance since, if the β-hydrogen exchange is due to a simple chemical tautomerization of pyruvate or a pyruvate derivative as suggested by Harley-Mason (1961), the increased alkalinity should greatly increase the β-hydrogen exchange.

Enzymatic Exchanges with Other Amino Acids. Figure 6 shows the NMR spectra of D_2O–glycine exchange as catalyzed

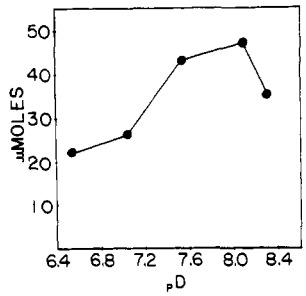

FIGURE 5: The pD profile of GPT-catalyzed exchange activity of L-alanine. The reaction mixture contained 100 nmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer of appropriate pD, 0.5 μmol of sodium pyruvate, 9.3 units of GPT, and 100 μmol of L-alanine in a total volume of 0.46 ml. The areas under the peaks obtained on a Varian A-60 NMR spectrometer were calculated by a planimeter and thus the number of micromoles of $-CDCD_3$ of L-alanine formed after 15 min was determined.

Singlet and triplet centered at 3.56 ppm represent $-CH_2-$ and $-CHD-$ of glycine.

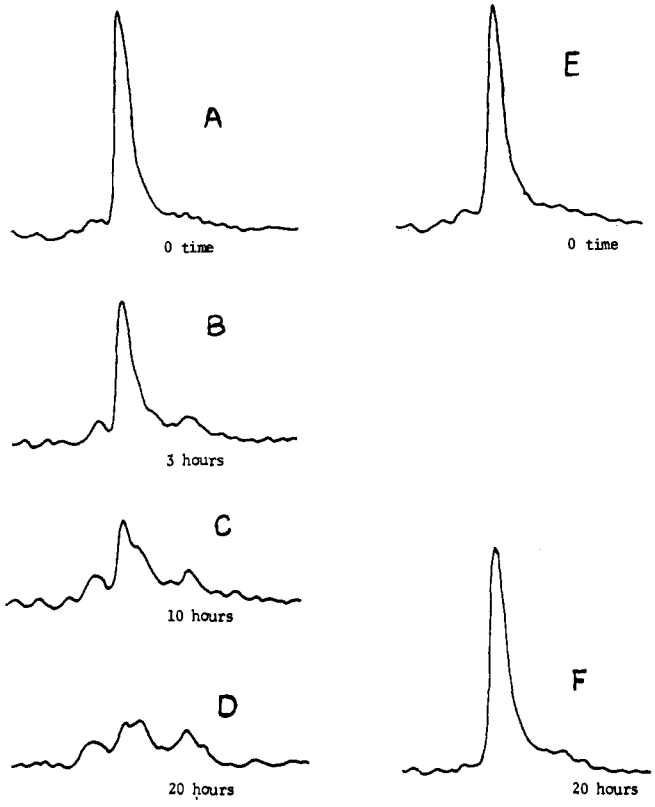

FIGURE 6: NMR spectra of D_2O–glycine exchange as catalyzed by GPT. The reaction mixture contained 100 nmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 9.3 units of GPT, and 100 μmol of glycine in a total volume of 0.46 ml. The spectra were taken on a Varian A-60 NMR spectrometer.

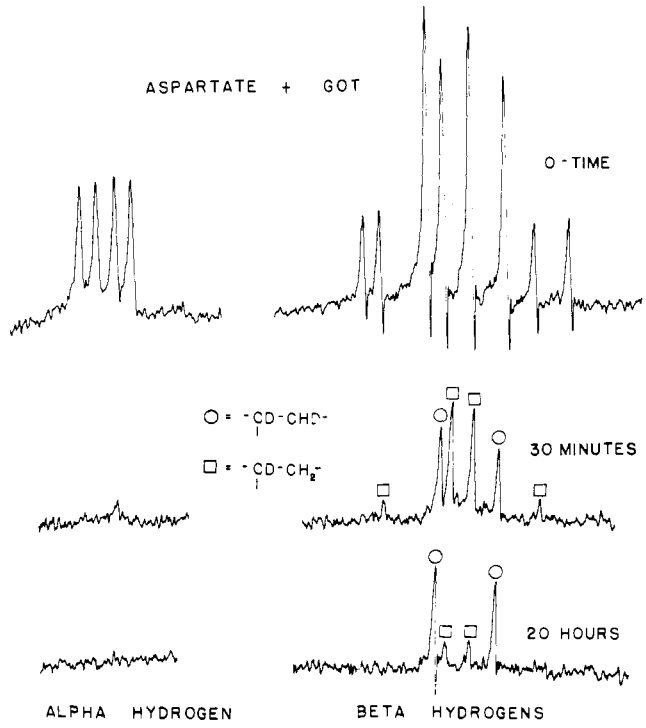

FIGURE 7: NMR spectra of L-aspartate with GOT. The reaction mixture contained 100 nmol of pyridoxal phosphate, 100 μmol of potassium phosphate buffer (pD 7.9), 2.0 μmol of sodium oxalacetate, 18.0 units of GOT, and 100 μmol of L-aspartate in a total volume of 0.46 ml. The spectra were taken on a deuterium-decoupled Varian XL-100 NMR spectrometer.

by GPT. After 20 h the area under the peaks is one-half as much as the zero-time peak, indicating that only one hydrogen of glycine was exchanged with D_2O. These spectra were taken on a Varian A-60 NMR spectrometer which is not deuterium decoupled; thus, the triplet is due to the coupling of the deuterium with the remaining proton. The enzymatic exchange of glycine was not stimulated by any added pyruvate and the rate that was observed was slower but comparable to the rate observed with L-alanine without any added pyruvate. Besmer and Arigoni (1968) observed that GPT catalyzes an exchange of glycine with D_2O to form (*R*)-glycine-d_1. Our NMR studies confirm the exchange of only one α hydrogen of glycine and provide a convenient method of following the time course of the reaction.

Figure 7 shows that GOT catalyzes the exchange of both the α and the β hydrogens of L-aspartate. The rate of exchange of α hydrogens is much faster than that of β hydrogens with D_2O. Oxalacetate shows a very fast (minutes) nonenzymatic exchange with D_2O and, hence, our experiments do not exclude the possibility of the nonenzymatic β exchange of oxaloacetate followed by transamination. GPT also causes a much slower exchange of both the α and the β hydrogens of L-aspartate. GPT did not significantly alter the nonenzymatic exchange of α-ketoglutarate with D_2O. Both GPT and GOT catalyze a rapid exchange of the α hydrogen of L-glutamate.

The following L-alanine analogues showed no exchange or substrate activity with GPT: D-alanine, β-alanine, DL-alaninol, L-serine, L-valine, isopropylamine, β-chloro-DL-alanine, taurine, and 1-aminoethylphosphonic acid. The only compounds that showed significant exchange properties are L-alanine, glycine, pyruvate, fluoropyruvate, L-aspartate, and L-glutamate. These studies confirm the observations of Saier and Jenkins (1967) on the specificity of GPT. Though glycine shows no transamination activity (Saier and Jenkins, 1967), it does indeed show significant exchange properties with GPT.

Manning et al. (1974) have shown that β-chloro-D-alanine inhibits alanine racemase from *Escherichia coli* and D-glutamate D-alanine transaminase from *Bacillus subtilis*. Our results show that β-chloro-DL-alanine inhibits the D_2O–alanine exchange reaction catalyzed by alanine racemase from *B. subtilis* (Babu and Johnston, 1974) and GPT from pig heart. In the presence of β-chloro-DL-alanine there was neither en-

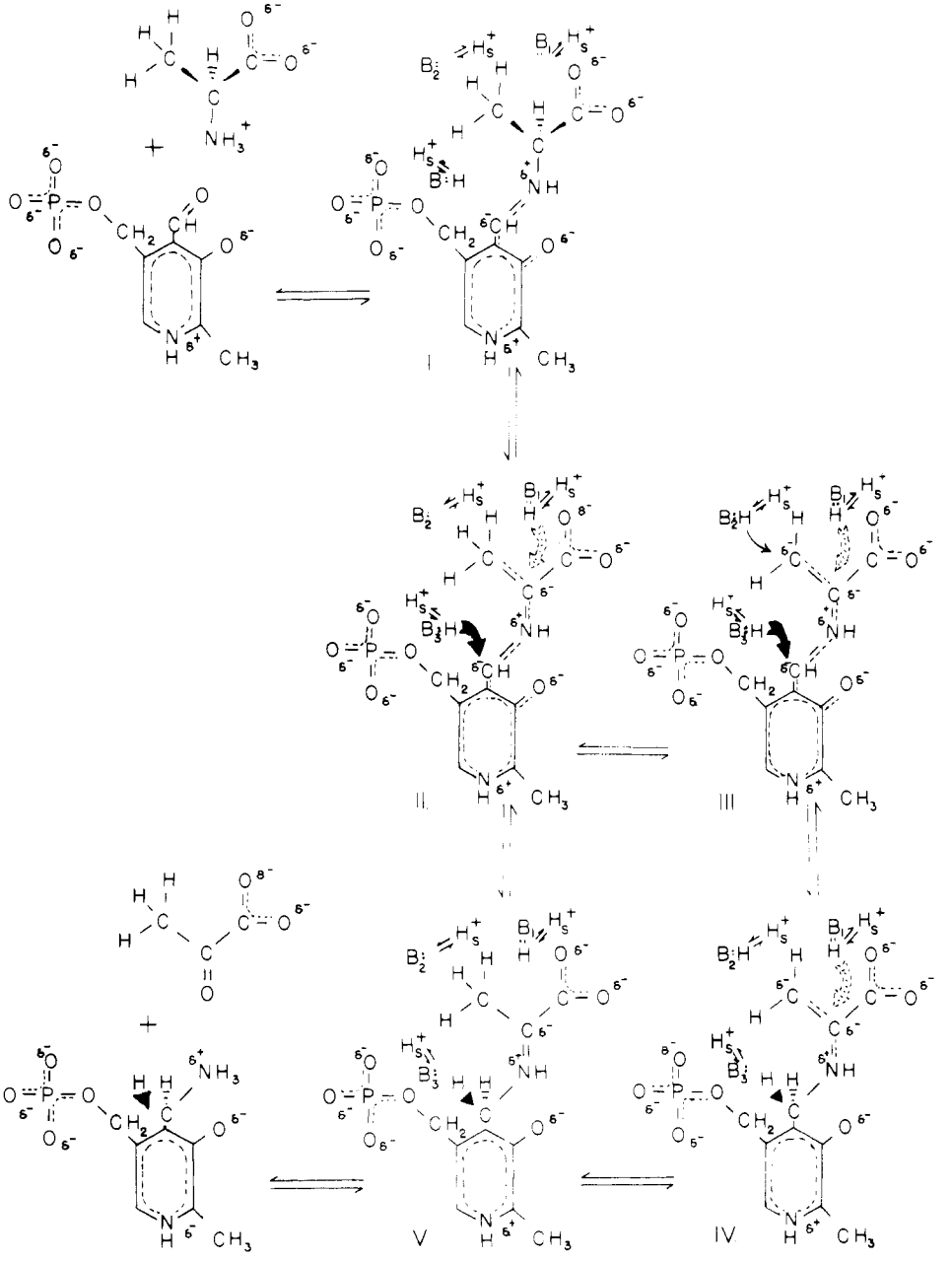

FIGURE 8: Proposed mechanism for GPT-catalyzed transamination reaction for L-alanine.

zymatic exchange of the α nor the β hydrogens of L-alanine by GPT.

Discussion

GPT catalyzes the exchange of α and β hydrogens of L-alanine at approximately the same rate. All possible combinations of exchanges at the β carbon are observed at very early stages. Consequently, multiple exchanges can occur by a single interaction of the substrate with the enzyme. Species in which exchanges have occurred at the β but not the α and at the α but not the β position can be observed. Since the relative rates of exchange of the basic groups on the enzyme with the solvent are not known, it is not possible to distinguish between the following two alternatives for these independent exchanges: (1) The exchanges can occur independently at the two positions or (2) the proton can be exchanged from the amino acid onto the active site of the enzyme and back to the substrate at one position, while the other position is exchanging with D_2O. The first of these alternatives seems unlikely since mechanistically the removal of the α hydrogen should precede the removal of the β hydrogen. Therefore, we prefer the second alternative for the interpretation of these data. Kinetic isotope studies by Cooper (1976) which show the removal of the α hydrogen is rate limiting support this conclusion.

The transaminase activity of the enzyme at the concentration of enzyme and under the conditions of the exchange reaction demonstrates that under condition of the exchange the enzyme is capable of reacting with the 100 μmol of substrate in less than 11 min. In the exchange reactions only one-half of the transaminating system is present and thus there is no decrease of substrate concentration during the exchange. The exchange is expected to be somewhat slower. However, under the optimum conditions with L-alanine the rate of exchange is of similar magnitude with the transamination rate. Cooper (1976) has shown the rate of exchange is the same as the pyruvate–alanine exchange catalyzed by the enzymes.

The exchange reaction is enzyme catalyzed and represents an inherent property of the enzyme–substrate complex. The rate of nonenzymatic exchange of pyruvate, even when excess of pyridoxamine phosphate was added, followed by transamination is too slow to explain the rapid enzymatic exchange seen in L-alanine.

The exchange is markedly stimulated by the addition of catalytic amounts of the copartner of the transamination reaction. Other substrates may show a stimulatory effect but in all cases examined it would appear that they exert their effects by forming the copartner by transamination. The precise reason for the stimulation is not clear but it is well known that transamination occurs by a ping-pong type mechanism which requires two distinct forms of the enzyme and the existence of the copartner allows both forms of the enzyme to be present at all times and both forms of the enzyme may be required for the rapid exchange.

Both GPT and GOT catalyze the exchange of the α hydrogens of glutamate and aspartate with the media. However, considerable confusion exists in the literature as to the nature and extent of enzyme-catalyzed exchange of the β hydrogens of these substrates by these enzymes. The rate of β exchange with aspartate is so extremely slow that this property of the enzyme cannot be an essential feature of the transaminase reaction. Optimum conditions of stimulation by copartner are required to observe the exchange; for example, Walter et al. (1975) observed no β exchange of aspartic acid when α-ketoglutarate is used as the keto acid, while Cooper (1976) confirmed our results (Babu et al., 1974) that the β hydrogens of aspartate exchange when oxaloacetate is used as a copartner. The results reported here demonstrating that the keto acid with the corresponding structure to the amino acid is more effective in promoting the β exchange readily explain why different results are obtained when the aspartate exchange is promoted by oxalacetate rather than α-ketoglutarate. Since we observe a very rapid nonenzymatic exchange of the hydrogens of oxaloacetate, the simpliest explanation of the observed exchange with aspartate is due to transamination with nonenzymatically exchanged oxaloacetate. This is in agreement with the proposal by Cooper (1976).

The labilization of the β hydrogens of the substrate by transaminases requires a specific interaction of the enzyme with β carbon of the substrate. This implies an extension of the conjugated system proposed for transaminase reaction (Braunstein and Shemyakin, 1953; Metzler et al., 1954) to include the α and the β carbons. Figure 8 shows our proposed mechanism to explain the properties of the enzyme–substrate complex. The relative significance of the contributions by the various structures indicated in the mechanism to the catalytic process of transamination cannot be ascertained by our experiments. The reactions shown in Figure 8 do not explicitly show the interaction of the coenzyme with the lysine residue on the enzyme, although this is known to be the donor of the pyridoxal in the transaminase reaction.

The equilibrium between structures 4 and 5 and/or structures 2 and 3 can account for the β exchange at the active site. The interconversion of structures 4 and 5 can occur nonenzymatically and thus can make a contribution to the observed β exchange. It is difficult to demonstrate the nonenzymatic interconversion of structures 1 and 2. The enzyme-catalyzed exchange with L-alanine at the α and the β positions, which would require at least the interconversion of structures 1, 2, and 3, occurs more rapidly than with the enzymic exchange of pyruvate. This suggests that the intermediate that is responsible for the exchange is more closely related to L-alanine than pyruvate. This implies that structures 1, 2, and 3 may be important in the exchange and that the exchange can occur prior to the protonation of the formyl carbon of the coenzyme. With substrates other than alanine, the existence of a side chain may restrict the position of the β hydrogens preventing them from occupying a position necessary to undergo rapid exchange.

While the racemase requires interaction of a basic group on the enzyme only at the α position (Babu and Johnston, 1974), the transaminase interacts with protons at three distinct positions designated as B_1, B_2, and B_3. The elegant studies of Cooper (1976) using α- and β-labeled alanine establish that B_1 and B_2 are acting independent of each other. This implies that the substrate is held more rigidly in the case of transaminase which may account for its specificity for only one enantiomer. The importance of rigid planar structures is suggested in the mechanism of action of transaminases proposed by Ivanov and Karpeisky (1969). It is attractive to postulate that pyridoxal phosphate requiring enzymes in the development of their various catalytic centers may have undergone an evolutionary process which started with the simple alanine racemase which extends the conjugated system only to the α hydrogen and ends with the mechanistically more complex homocysteine and homoserine enzymes where the conjugated system is extended to the γ carbon. The interaction of alanine with the transaminase represents an enzyme property which is not only characteristic of the transamination but also shows some of the properties of pyridoxal phosphate enzymes which labelize a bond on the β carbon.

While these studies allow a more accurate representation of the nature of the enzyme–substrate interaction with alanine, it is difficult to ascertain the relative importance of each of all the possible interactions in the catalytic mechanism since the information is not yet available as to the relative rates of exchange of the basic groups on the enzyme with protons from the substrate and from the solvent.

These NMR studies can also be used successfully to assay both the enzyme and the substrate. Such NMR assay for alanine racemase and its substrates have been reported from this laboratory (Babu et al., 1975). This type of assay procedure requires a single incubation and the kinetics of the reaction can be easily followed during the progress of the reaction. The NMR exchange studies provide a convenient tool for determining the specific properties of one-half of a transamination reaction independent of the other half.

Acknowledgment

We thank Mr. A. E. Sopchik for his technical assistance in obtaining the NMR spectra data. We are grateful for the fruitful discussions with Dr. G. A. Vidaver.

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Intact Photoreceptor Membrane from Bovine Rod Outer Segment: Size and Shape in Bleached State†

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ABSTRACT: Photoreceptor disk membranes isolated from bovine rod outer segments are suspended in dilute aqueous sucrose (4.36×10^{-3}%) and bleached, and their size and shape are determined with quasielastic and elastic light scattering. They are found to be extremely homogeneous spherical vesicles with a radius of 0.49 ± 0.07 μm which appear to have derived from the native disk shape by osmotic swelling.

The visual process involves complex sequences of events in transducing photochemical energy to electrical energy (Wald, 1968; Rodieck, 1973; Kliger and Menger, 1975). A vast literature has accumulated delineating the role of the visual pigment membrane in this process (Hagins, 1972; Abrahamson, 1975). Our goal is to isolate disk membranes from vertebrate rod outer segments (ROS[1]) as intact as possible and focus on their static and dynamic structure vis-à-vis the photoreceptor function.

This report represents a preliminary step toward that end: namely, the characterization of photoreceptor membranes suspended in dilute aqueous sucrose. Their size and shape are determined from quasielastic and elastic light-scattering measurements.

Experimental Procedure

Material. Except for some minor changes, the procedure of Smith et al. (1975) was used for the isolation and purification of bovine ROS membranes. For each preparation, 2–3 g of frozen retinae (Hormel Co.) was thawed with stirring in 9 ml of 36% sucrose–buffer (0.068 M sodium phosphate buffer, pH 7) for 30 min at 4 °C. The resulting suspension was diluted to 18 ml with 36% sucrose–buffer, passed through a wide-gauge syringe needle two to three times to effect complete suspension, layered with 15 ml of buffer, and then centrifuged for 1 h at 25 000 rpm in an SW-27 rotor (Beckman, Model L5-50 centrifuge). The crude outer segments that floated to the sucrose–buffer interface were harvested with a syringe, suspended in buffer, and pelleted by centrifugation at 15 000 rpm for 20 min. The supernatant was decanted and the pellet was resuspended in 36% sucrose–buffer. The ROS were floated again, and the carpet (the material which collected at the sucrose–buffer interface) was divided into two parts for the second washing in buffer. Each of the purified pellets was gently rinsed with 5% aqueous Ficoll (molecular weight 400 000; Sigma) to minimize the amount of remaining buffer in the tube and finally suspended in 17 ml of 5% Ficoll. Dust-free nitrogen gas was bubbled through the Ficoll suspensions and they were left standing overnight in the dark at 4 °C to allow complete bursting of the rods. The ROS disk membranes were harvested from the surface of each Ficoll solution after centrifugation at 25 000 rpm for 2 h. All the above operations were performed under a dim red light (Kodak No. 1 filter, 15-W bulb) at 4 °C. To check the purity of the ROS disk membranes so obtained, the absorbances A_{498} at 498 nm and A_{278} at 278 nm were measured on a Gilford spectrophotometer. The ratios A_{278}/A_{498} were substantially constant (2.46 ± 0.23) for the five independent preparations (see below), indicating that our preparations were reasonably pure (Raubach et al., 1974a; Smith et al., 1975).

Quasielastic Light Scattering. The instrument, the data acquisition scheme, and the spectrum analysis method employed here have been described elsewhere (Shaya et al., 1974).

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[1] Abbreviations used: ROS, rod outer segment; QLS, quasielastic light scattering; ELS, elastic light scattering.