Transfer of C^{α}-Hydrogen of Glutamate to Coenzyme of Aspartate Aminotransferase during Transamination Reaction†

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ABSTRACT: During the transamination reaction of mitochondrial aspartate aminotransferase, transfer of tritium from the α-position of glutamate to the *pro-S* position of C4′ of pyridoxamine 5′-phosphate was detected. A fast mixing and quenching device had to be used in order to reduce the number of transamination cycles undergone by the enzyme and thus to minimize the accompanying exchange of label with water. The extent of transfer of label (mean value 1.5%; range 0.8–4%) indicates that the 1,3-prototropic shift follows a stepwise rather than a concerted mechanism and that a single acid/base group is responsible for the proton transfer. The actual extent of proton transfer has to be much higher because the rate of α-tritium exchange with solvent was only ~10% of that of the turnover of unlabeled substrate, reflecting either an isotope effect or a retention of the tritium label in the reaction center during tautomerization. Under the assumption of an isotope effect, the actual transfer may be estimated to be 13%. This value is consistent with the notion of Lys-258 acting as the proton transferring group in which case the maximal value of transfer in an active site not accessible to solvent during the 1,3-prototropic shift would be 33%. However, alternative mechanisms involving Tyr-70 or a water molecule enclosed in the active site serving as acid/base group cannot be excluded on the basis of the present results. Furthermore, in these investigations aspartate aminotransferase was found to catalyze also the exchange of tritium from the β-position of glutamate, though at a rate 350 times slower than that of the α-exchange.

The critical step that distinguishes enzymic transamination from other pyridoxal-dependent reactions is the tautomerization of the external aldimine to the ketimine. Ayling et al. (1968) and Dunathan (1971) found 2–5% transfer of α-deuterium from alanine to the C4′ position of pyridoxamine when the transamination of alanine and pyridoxal was catalyzed by pyridoxamine-pyruvate transaminase in H_2O. If the reaction was carried out in D_2O, 50% of α-hydrogen of the same amino acid was transferred. On the basis of these results, a single acid/base group of the enzyme was proposed to be responsible for the elimination of the α-hydrogen from the amino acid substrate and its subsequent addition at the C4′ position. However, a similar procedure for detecting proton transfer failed in the case of aspartate aminotransferase (Dunathan, 1971).

In this paper, transfer of tritium from the α-position of glutamate to the C4′ position of pyridoxamine 5′-phosphate is demonstrated, and the data are discussed in terms of the active site group (Ford et al., 1980; Arnone et al., 1982; Harutyunyan et al., 1982; Kirsch et al., 1984) mediating the prototropic shift.

Contradictory reports about the occurrence of β-hydrogen exchange from glutamate in the presence of aspartate aminotransferase have appeared (Walter et al., 1975; Cooper, 1976). The results given in this paper clearly demonstrate β-exchange.

Experimental Procedures

Materials. The mitochondrial isoenzyme of aspartate aminotransferase (sp act. 240 units/mg, 25 °C) was isolated from chicken heart (Gehring et al., 1977b). The apoenzyme of the α subform of the cytosolic isoenzyme from pig heart was prepared according to Schlegel & Christen (1974). Enzymic activity was measured in the coupled assay with malate dehydrogenase (EC 1.1.1.37; obtained from Boehringer) as described previously (Birchmeier et al., 1973) except that the concentrations of aspartate and 2-oxoglutarate were 20 mM. Alkaline phosphatase from calf intestine was from Boehringer. Aspartic acid, 2-oxoglutaric acid, and L-glutamic acid were from Fluka; pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, pyridoxal, pyridoxamine, and oxalacetic acid were from Merck. L-[G-^{3}H]Glutamic acid (34–43 Ci/mmol) labeled in α- and β-positions was from Amersham.

Determination of Rate of α- and β-Exchange. The reaction mixture (2 mL) containing radioactive glutamate (10 mM, 1 μCi/μmol), 2-oxoglutarate (1 mM), and 5 mM sodium phosphate (pH 7.5) was lyophilized 3 times in order to remove sublimable counts. An aliquot of 100 μL was taken for determining the initial value. The exchange (at 25 °C) was started by addition of mitochondrial aspartate aminotransferase at various concentrations (7–200 μg/mL). At different times, aliquots of the reaction mixture (100 μL) were added to 100 μL of 0.1 M HCl and immediately frozen in order to quench the reaction. The sublimable radioactivity and the residue were counted with Aquassure (from New England Nuclear; maximal counting efficiency was 43%).

Tritium Transfer from Tritiated Glutamate to Pyridoxamine 5′-Phosphate. Enzyme (8–10 mg/mL, 1.2 mL) in 5 mM sodium phosphate (pH 7.5) was mixed at room temperature with an equal volume of 10 mM glutamate (11.6 μCi/μmol)–5 mM sodium phosphate (pH 7.5) in a stopped-flow apparatus (Durrum D-130). The stop syringe was replaced by a regular glass syringe, filled with either 2 mL of trichloroacetic acid (23%, w/v) or 2 mL of 1 M HCl. Before use, the solution of radioactive glutamate was lyophilized in order to remove tritiated water. When trichloroacetic acid had been used, the quenched reaction mixture was centrifuged, and trichloroacetic acid in the supernatant was extracted with ether.

Isolation of Labeled Pyridoxamine 5′-Phosphate. The supernatant (see above) or the whole quenched reaction

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Table I: Demonstration of ^3H Transfer from α-Position of Glutamate to Cofactor

expt no.[a]	sp radioactivity at α-position [cpm μmol^{-1} ($\times 10^{-6}$)]	labeled pyridoxamine phosphate recovered		labeled pyridoxamine recovered		% transferred α-^3H
		cpm	μmol ($\times 10^3$)[b]	cpm	μmol ($\times 10^3$)[b]	
1	1.48	112	4.4			1.7
2		313	16.1			1.3
3–6	1.39, 1.49	377–445	28.9–35.2	321[c]	32.1[c]	0.9 (0.7)[c]
7	0.944			553	27.7	2.1
8				95	2.4	4.2
9				297	7.7	4.1
10	1.39			136 (30)[d]	7.9 (1.5)[d]	1.2 (1.4)[d]
11				143	13.0	0.8
12	1.49			209	16.0	0.9
13–14				223–253 (259)[d]	20.5–20.8 (23.4)[d]	0.75 (0.75)[d]

[a] Different batches of tritiated glutamic acid were used for the following experiments: 1 and 2; 3, 4, 10, and 11; 7–9; 5, 6, and 12–14. [b] The differences in the indicated recovery of cofactor are due to using up part of the material in test runs. [c] Purified pyridoxamine phosphate was treated with alkaline phosphatase, and the pyridoxamine generated was again subjected to high-voltage electrophoresis. [d] Purified pyridoxamine was subjected to paper chromatography. Detailed conditions and descriptions are given under Experimental Procedures and Results.

mixture (if HCl had been used as quencher) was sublimed for measuring the exchanged counts. The residue was dissolved in 500 μL of 0.3 M acetic acid–0.3 M pyridine and applied to a DEAE-52 cellulose column (0.9 × 17 cm) equilibrated and eluted (~8 mL/h) with the same buffer (see Figure 2). The samples obtained with HCl as quencher had to be centrifuged before they were applied to the column in order to remove precipitated protein. The fractions containing pyridoxamine 5′-phosphate were pooled and lyophilized. The residue was dissolved with 100 μL of water and subjected to two-dimensional high-voltage paper (Whatman No. 3MM) electrophoresis at pH 3.5 (pyridine–acetic acid–water, 1:10:89) and at pH 1.9 (acetic acid–formic acid–water, 4:1:45). Pyridoxamine 5′-phosphate was identified (Gehring et al., 1977a) and eluted from the paper with 1 mL of 0.1 M HCl, its absorption spectrum recorded, and the radioactivity measured with Aquassure.

Isolation of Labeled Pyridoxamine. The pH value in the supernatant obtained after addition of trichloroacetic acid (see above) was adjusted to ~8.5 with sodium hydroxide. The supernatant was treated with alkaline phosphatase (2 units/mL) for 20 min at room temperature, ultrafiltrated (Centriflo CF25, Amicon), and sublimed. The residue was dissolved in 500 μL of 10 mM potassium phosphate (pH 5.6) and applied to an RP-18 column (see Figure 3). The elution rate was 1.3 mL/min. The fractions containing pyridoxamine were pooled, lyophilized, subjected to high-voltage paper electrophoresis at pH 1.9, and analyzed as described for pyridoxamine 5′-phosphate. In the experiment in which the reaction had been stopped with HCl, the mixture was immediately sublimed, the residue dissolved in 2 mL of water, the pH adjusted to ~8.5, and the precipitated protein removed by centrifugation before the solution was treated with alkaline phosphatase.

Analysis of Tritium Distribution at C4′ of Pyridoxamine. The procedure used was essentially that described previously (Dunathan et al., 1968; Zito & Martinez-Carrion, 1980). Purified labeled pyridoxamine (8 nmol) was incubated with apoenzyme of cytosolic aspartate aminotransferase from pig heart (3.5 mg/mL), glutamate (3 mM), and 2-oxoglutarate (0.1 mM) in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (final volume 0.1 mL) at 37 °C, pH 8, for 15 h. The reaction was stopped by freezing, and the sublimable radioactivity was measured.

Results

Release of Tritium from α- and β-Positions of Glutamate. The ^3H occupancy at the α- and β-positions of commercially available labeled glutamic acid is not routinely determined by the suppliers and had to be estimated experimentally. On request, the supplier gave an estimate of about 8% in the α-position and 92% in the β-position determined once by NMR measurements. In our experience (see below), the values differed somewhat from batch to batch. In order to estimate the percentage of label at the α-position of glutamate, a mixture of the tritiated glutamate and unlabeled 2-oxoglutarate was incubated with aspartate aminotransferase and the released radioactivity measured as a function of time. The detritiation follows biphasic kinetics (Figure 1). Both phases represent (for the first phase, corresponding to α-exchange, see insert of Figure 1) homogeneous first-order reactions. Extrapolation of the second phase, corresponding to β-exchange, to time zero gave the percentage of specific radioac-

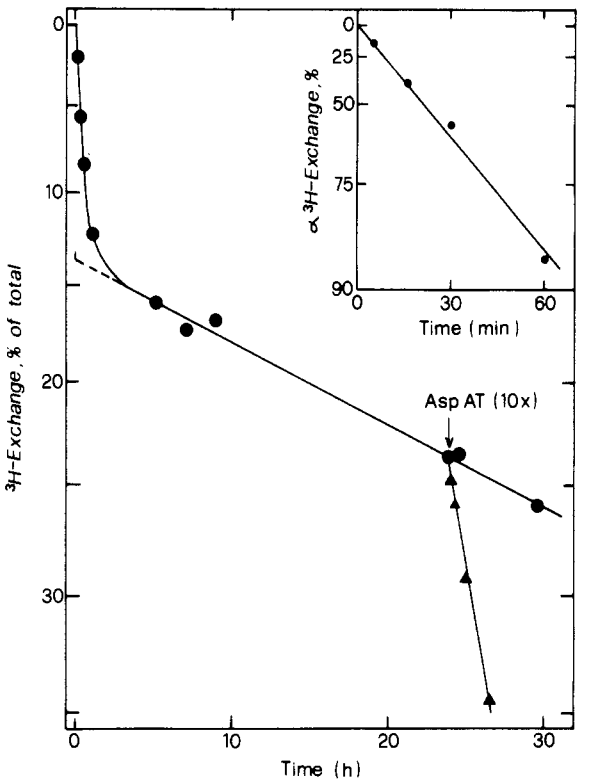


FIGURE 1: Semilogarithmic presentation of the release of tritium from the α- and β-positions of glutamate as a function of time. Mitochondrial aspartate aminotransferase (Asp AT, 20 μg/mL) was incubated in 5 mM sodium phosphate, pH 7.5, with radioactive glutamate (10 mM) and unlabeled 2-oxoglutarate (1 mM). In the insert, the first phase is replotted. At the time indicated by the arrow, the concentration of Asp AT was increased to 200 μg/mL.

Table II: Comparison of Enzymic Activity Measured by Consumption of Oxalacetate and by Exchange of α-^{3}H of Glutamate with Water

enzymic activity[a] (μmol min^{-1} mg^{-1}) measured by		
oxalacetate consumption (v_{OA})	exchange of α-^{3}H of glutamate with water ($v_{^3H}$)	($v_{^3H}/v_{OA}$)
50	5.2[b]	10.4 (9.2–12.5)[c]

[a] With 5 mM glutamate– 1 mM oxalacetate in 50 mM sodium phosphate, pH 7.5. [b] Mean value of four experiments (SD ±0.5). [c] Extreme values of four experiments. Experimental details are described under Results.

tivity contributed by the α-position. The estimated values were 9–15% in the four batches of labeled glutamate used (the specific radioactivities are listed in Table I). The rates of both phases depend on the enzyme concentration (see, as an example, the second phase in Figure 1). The second-order rate constants obtained from plots as in Figure 1 at different enzyme concentrations (7–200 μg/mL) are for the α-elimination 78.5 min^{-1} mM^{-1} (SD ±5.0 min^{-1} mM^{-1}, n = 6) and 0.22 min^{-1} mM^{-1} (SD ±0.03 min^{-1} mM^{-1}, n = 13) for the β-exchange, respectively.

Rapid Mixing and Quenching. Pyridoxal phosphate and pyridoxamine phosphate do not dissociate from aspartate aminotransferase during the catalytic cycle. A continuous labeling of the cofactor in the presence of a catalytic amount of enzyme as in the case of pyridoxamine-pyruvate transaminase (Ayling et al., 1968) or aspartate β-decarboxylase (Chang et al., 1982) is therefore not possible. In order to obtain labeled pyridoxamine phosphate in amounts sufficient for analysis, a high enzyme concentration had to be used. This condition required a fast mixing and quenching device (see Experimental Procedures) in order to avoid extensive exchange of tritium with water (see Figure 1) with the concomitant decrease in specific radioactivity at the α-position of glutamate. Mitochondrial aspartate aminotransferase in the pyridoxal phosphate form was mixed with labeled glutamate. A minimal reaction time of 10 ms was estimated from the technical specifications of the stopped-flow apparatus without considering the time required for quenching. The half-life for the conversion to the pyridoxamine form as estimated from the rate given in Table II is ~20 ms. The actual reaction time must be longer than 10 ms and probably considerably more than 20 ms because the ratio of pyridoxamine phosphate to pyridoxal phosphate had reached already the constant equilibrium value of ~2.3. Exchange of label with water was always examined and was in the range of 0.5–1% of the total radioactivity. From the kinetics of the exchange (Figure 1), one has to assume the sublimable radioactivity to originate almost exclusively from the α-position. The percentage of the radioactivity at the α-position of glutamate was thus diminished maximally by the indicated values.

After the reaction was quenched by acid (see Experimental Procedures), the dissociated cofactors were purified by two different procedures. In the first, pyridoxamine phosphate was isolated by ion-exchange chromatography and two-dimensional high-voltage paper electrophoresis (see below). In the second, the phosphate moiety was cleaved off by alkaline phosphatase and the resulting pyridoxamine purified by HPLC, high-voltage paper electrophoresis, and paper chromatography (see below).

Isolation and Analysis of Labeled Pyridoxamine Phosphate. The sublimable counts in the supernatant from the quenched

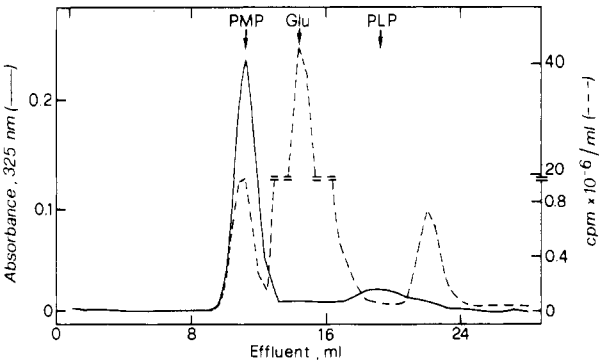


FIGURE 2: DEAE-cellulose chromatography of the reaction mixture. The elution volumes for pyridoxamine phosphate (PMP), glutamate, and pyridoxal phosphate (PLP) are indicated by arrows. The preparation of the sample and conditions for elution are detailed under Experimental Procedures.

reaction mixture were trapped and measured in order to check for exchanged counts (see above). The supernatant dried by sublimation was redissolved and applied to a DEAE-cellulose column (Figure 2 shows a typical elution pattern). Pyridoxamine phosphate cochromatographed with a radioactive peak. Glutamate was eluted between pyridoxamine phosphate and pyridoxal phosphate. The latter was followed by a very small unidentified radioactive peak (presumably 2-oxoglutarate formed during the reaction). The fractions containing pyridoxamine phosphate were pooled and subjected to two-dimensional high-voltage paper electrophoresis (pH 3.5 and 1.9). The contaminating radioactivity that could be removed by this procedure was already present in the batches of glutamic acid. Pyridoxamine phosphate was visualized on the paper by its fluorescence and eluted with 0.1 M HCl. Above and below the fluorescent spot additional paper strips were cut out and eluted, and the radioactivity of the eluate was counted in order to assure that no radioactive material was overlapping with the spot of pyridoxamine phosphate. A piece of paper adjacent to the pyridoxamine phosphate spot and identical in size was eluted and used as a blank. The spectra of the eluate corresponded to that of pyridoxamine phosphate. The yield after the whole purification procedure was ~30%. After the concentration of the isolated cofactor (ϵ_{293} = 9 mM^{-1} cm^{-1}; Peterson & Sober, 1954) was estimated, the radioactivity of the solution was measured. The specific radioactivity was compared with that of the α-position of glutamic acid (Table I). In order to verify the purity of pyridoxamine phosphate, purified material of experiments 3–6 was pooled and incubated with alkaline phosphatase and subsequently subjected to the same high-voltage paper electrophoresis (pH 1.9). The resulting pyridoxamine was eluted and analyzed as just described. The percentage of specific radioactivity incorporated (Table I) was slightly decreased. This is probably due to the mainly acidic conditions during this step of purification, which might favor exchange of the C4′-hydrogens of the cofactor with solvent (Dunathan et al., 1968).

Purification and Analysis of Labeled Pyridoxamine. The acid-dissociated cofactors were treated with alkaline phosphatase and then subjected to reverse-phase HPLC (see Experimental Procedures). A typical elution pattern is shown in Figure 3. In this chromatography, glutamate appeared first, reflected by the huge radioactive peak. Pyridoxamine was eluted later, followed by pyridoxal. The fractions containing pyridoxamine were further purified by high-voltage paper electrophoresis (pH 1.9). The fluorescent spots were eluted and analyzed (ϵ_{293} = 8.5 mM^{-1} cm^{-1}; Peterson & Sober, 1954) as described for pyridoxamine phoshate. The calculated

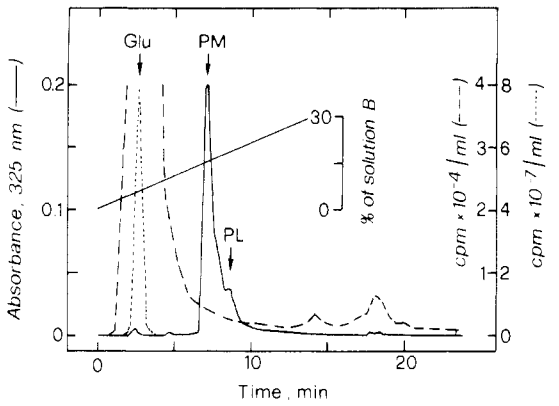


FIGURE 3: Reverse-phase HPLC of the reaction mixture treated with alkaline phosphatase (see Experimental Procedures). The column (RP-18) was equilibrated with buffer A (10 mM potassium phosphate, pH 5.6). The sample was eluted by applying a gradient of solution B (80% methanol–20% water). The elution times of glutamate, pyridoxamine (PM), and pyridoxal (PL) are indicated by arrows.

specific radioactivity of pyridoxamine is given in percentage of that of the α-position of glutamate (Table I). Additional purification by paper chromatography (Chang et al., 1982) did not change the values (see footnote *d* of Table I).

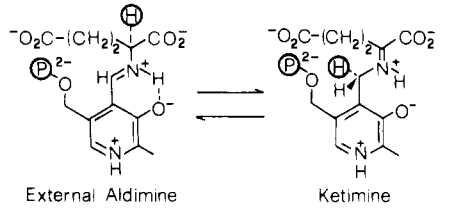
Half of the purified pyridoxamine from experiment 12 was further analyzed for its tritium distribution by incubation with cytosolic apo aspartate aminotransferase from pig. This enzyme has been shown to catalyze stereospecifically the exchange of the *pro-S* hydrogen at C4′ of pyridoxamine with solvent (Dunathan, 1971). After the reaction had been stopped by freezing, 82% of the radioactivity of pyridoxamine was sublimable (for detailed conditions, see Experimental Procedures) and therefore had been exchanged with solvent. Thus, one can conclude that the label was indeed in the *pro-S* 4′-position of the cofactor.

Comparison of Enzymic Activities Determined by Consumption of Oxalacetate and Tritium Exchange. With tritiated glutamate as substrate, the amount of tritium released into water was measured, and the rate of exchange was compared with the rate of substrate consumption. The concentration of glutamate (5 mM, 9.1 μCi/μmol) was the same as that in the fast mixing experiments. The concentration of oxalacetate (1 mM) corresponded to saturation conditions. The consumption of oxalacetate was measured by monitoring the decrease in absorbance of oxalacetate (ϵ_{280} = 0.57 mM^{-1} cm^{-1}; Velick & Vavra, 1962). From the same assay mixture, five aliquots (200 μL each) were taken in 1-min intervals, and the reaction was stopped by HCl (0.1 M) and subsequent freezing. By counting the sublimable radioactivity and by using the specific radioactivity at the α-position, the apparent enzymic activity with tritiated glutamate was estimated. These rates are listed in Table II.

Discussion

1,3-Prototropic Shift. The present results demonstrate for the first time that during the tautomerization of the external Schiff base (Scheme I) in an aminotransferase with nondissociable cofactor the α-proton (encircled in Scheme I) of the substrate is transferred to the *pro-S* position at C4′ of the cofactor. The observed transfer (mean value 1.5%; range 0.8–4%; Table I) is comparable to that observed with pyridoxamine pyruvate transaminase (Ayling et al., 1968; Dunathan, 1971) where the cofactor, pyridoxamine or pyridoxal, is itself a substrate and dissociates from the enzyme. Analogous but higher transfer (17%) from aspartate to pyridoxamine phosphate had been found with aspartate β-decarboxylase in the abortive decarboxylation–transamination reaction yielding pyruvate (Chang et al., 1982). This reaction is very slow when compared to the regular β-decarboxylation, and since proton addition at C4′ is not an obligatory step in the β-decarboxylation reaction, one could imagine that the mechanism evolved for transamination in this enzyme might differ from that of the normal transamination reaction. The conservation of the proton, however, indicates a common mechanism for the 1,3-prototropic shift in pyridoxal phosphate enzymes, as already suggested by Dunathan & Voet (1974).

Scheme I: 1,3-Prototropic Isomerization



The measured transfer of label in the case of aspartate aminotransferase is a strong indication for a stepwise rather than a concerted mechanism of tautomerization. The fact that the percentage of tritium appearing in pyridoxamine phosphate is small should not detract from the importance of this result. Several explanations are possible for the low percentage of tritium transfer. The group that accepts the label from the α-carbon may, besides adding the label to C4′ of the coenzyme, exchange it with solvent, either directly or via other groups of the enzyme. From the present results it is obvious that the reaction center is hardly accessible to solvent during tautomerization; otherwise, no transfer of the label on the coenzyme would be detectable. Crystallographic studies have shown that there is room for only two or three water molecules in the active site when a substrate molecule is presesnt (Kirsch et al., 1984). In addition, a primary isotope effect on the prototropic shift could be responsible for an apparently low efficiency of the tritium transfer. With α-deuterated glutamate and cytosolic aspartate aminotransferase, an isotope effect on V_{max} of about 2 was measured (Fang et al., 1970). The effect with tritiated substrate is expected to be higher according to the relationship defined by Swain et al. (1958). The 10-fold slower exchange of α-tritium with solvent in comparison to the rate of oxalacetate consumption (Table II) may reflect an isotope effect of this magnitude. The slow exchange may also be due to a shuttle of the proton between C$^{\alpha}$ of the substrate and C4′ of the cofactor (see Scheme I), releasing a proton into the solvent only every tenth cycle. A further possibility could be that the hydrogen at C4′ is more labile in the enzyme-bound than in the unbound state. Thus, its exchange could occur and might even be accelerated during the acid denaturation of the enzyme used to stop the reaction. In the absence of the protein, the exchange of the C4′-hydrogen is very slow (Dunathan et al., 1968). An exchange during the quenching process, which is indeed the least controlled experimental step (i.e., the reproducibility of the mixing process), could account not only for the low percentage of transfer but also for the variation of the values in different experiments.

In any case, the actual transfer must be substantially higher than the measured values. The total tritium released from the α position comprises the tritium exchanged with the solvent (~10%, Table II) and that retained on pyridoxamine phosphate (1.5%, Table I). Thus, the fraction of transferred tritium is 13% [1.5/(10 + 1.5)] of the total tritium released. This value is consistent with the notion of a lysine residue acting as the proton transferring group (Snell, 1962; Dunathan, 1971) in which case the maximal value of transfer in an active site

not accessible to solvent during tautomerization would be 33%. Possible reasons for lower values such as exchange of label to other groups of the enzyme, uncertainty in isotope effect, variation in reaction times, and uncertainties in quenching are discussed above. As shown by crystallographic studies, the only potential acceptor groups close enough to act as acid/base are the ϵ-amino group of Lys-258 and the phenolic hydroxyl group of Tyr-70 (Jansonius et al., 1984). Structural evidence (Jansonius et al., 1984; Kirsch et al., 1984) favors Lys-258. The present results are compatible with this hypothesis but of course do not eliminate an alternative mechanism with Tyr-70. The involvement of water molecules enclosed near to the reaction center (see above) and engaged in a proton relay system as proposed by Jenkins & Harruff (1979) could also account for a transfer of 50% or less.

By studying the isotope effects of deuterated solvent and of the deuterated substrate analogue *erythro*-3-hydroxyaspartate on the transamination reaction catalyzed by cytosolic aspartate aminotransferase from pig, Jenkins & Harruff (1979) suggested a group with two equivalent hydrogens, excluding a lysine residue, but could not eliminate the participation of a group with one acidic hydrogen. In the transamination reaction with 3-hydroxyaspartate, which is a very slow process, Tyr-70 may serve this function. Whether the conclusions obtained with 3-hydroxyaspartate are also valid for normal substrates is a matter of conjecture. The rate-limiting step might be different as indicated by the absence of an isotope effect on the overall reaction with 3-hydroxyaspartate (Jenkins & Harruff, 1979). With deuterated glutamate and cytosolic aspartate aminotransferase, however, an isotope effect of 2 was measured (Fang et al., 1970). This value was confirmed with mitochondrial isoenzyme and the effect on V_{max}/K_m was even higher (3.2; unpublished results).

The present data are compatible with the known stereochemical features of enzymic transamination. The conformation shown in Scheme I had been deduced from stereochemical [see Dunathan (1971)] and structural (Kirsch et al., 1984) consideratons. The detected transfer of tritium (encircled in Scheme I) requires a cis mechanism for the 1,3-prototropic shift. Both proposed acid/base catalysts (Lys-258 or Tyr-70) as well as enclosed water molecules are situated above the A face of the coenzyme (Ford et al., 1980; in Scheme I below the plane of the paper), i.e., on the *si* face with respect to C4′ (Kirsch et al., 1984). Aspartate aminotransferase catalyzes the exclusive removal of the *pro-S* hydrogen at C4′, which points to the side where the proposed catalytic groups are situated. The α-hydrogen is oriented on the same side (*si*) perpendicular to the coenzyme ring in accordance with Dunathan's (1971) postulate for the most favorable conformation for proton abstraction.

β-Exchange. The second slow phase of Figure 1 has to be ascribed to exchange of the β-hydrogens. This exchange depends on the concentration of the enzyme but is more than 300 times slower than that of the α-exchange. Apparently, β-exchange is a direct catalytic effect of mitochondrial aspartate aminotransferase and is not due to enolization of the keto acid product. The acceleration of exchange observed on addition of more enzyme cannot be explained by enolization of the keto acid for the following reasons: Under the conditions used (Figure 1), the concentration of the keto acid 2-oxoglutarate remained constant, and consequently, the concentration of enol could not change on addition of more enzyme. The equilibration of label between glutamate and 2-oxoglutarate was established before more enzyme was added as indicated by the total exchange of the α-hydrogen of glutamate (Figure 1). The estimated rate of β-exchange is so slow that, under the conditions used by Walter et al. (1975) and Cooper (1976) in their NMR studies, hardly any exchange could have occurred. This would explain why they have not observed β-exchange with deuterated glutamate. Walter et al. (1975) have observed exchange with tritiated glutamate. The magnitude of the rate of exchange that they found, however, corresponds rather with the rate of α-exchange. Apparently, their preparation of glutamate contained tritium label also in the α-position.

Acknowledgments

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Registry No. Hydrogen ion, 12408-02-5; tritium, 10028-17-8; glutamic acid, 56-86-0; pyridoxamine 5′-phosphate, 529-96-4; aspartate aminotransferase, 9000-97-9.

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Effects of *N,N'*-Dicyclohexylcarbodiimide and *N*-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline on Hydride Ion Transfer and Proton Translocation Activities of Mitochondrial Nicotinamidenucleotide Transhydrogenase†

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ABSTRACT: *N,N'*-Dicyclohexylcarbodiimide (DCCD) inhibits the mitochondrial energy-linked nicotinamidenucleotide transhydrogenase (TH). Our studies [Phelps, D. C., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 8217–8221; Phelps, D. C., & Hatefi, Y. (1984) *Biochemistry 23*, 4475–4480] suggested that the inhibition site of DCCD is near the NAD(H) binding site, because NAD(H) and competitive inhibitors protected TH against inhibition by DCCD and, unlike the unmodified TH, the DCCD-modified TH did not bind to NAD–agarose. Others [Pennington, R. M., & Fisher, R. R. (1981) *J. Biol. Chem. 256*, 8963–8969] could not demonstrate protection by NADH, obtained data indicating DCCD inhibits proton translocation by TH much more than hydride ion transfer from NADPH to 3-acetylpyridine adenine dinucleotide (AcPyAD), and concluded that DCCD modifies an essential residue in the proton channel of TH. The present studies show that *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) also inhibits TH. The inhibition is pseudo first order at several EEDQ concentrations, and the reaction order with respect to [EEDQ] is unity, suggesting that inhibition involves the interaction of one molecule of EEDQ with one active unit of TH. The EEDQ-modified TH reacts covalently with [^{3}H]aniline, suggesting that the residue modified by EEDQ is a carboxyl group. More significantly, it has been shown that the absorbance change of oxonol VI at 630 minus 603 nm is a reliable reporter of TH-induced membrane potential formation in submitochondrial particles and that TH-catalyzed hydride ion transfer from NADPH to AcPyAD and the membrane potential induced by this reaction are inhibited in parallel by either DCCD or EEDQ.

Vectorial proton translocation by the ATP synthase complexes of prokaryotic and eukaryotic organisms [for a review, see Senior (1983)], by complex III (ubiquinol–cytochrome *c* oxidoreductase) of bovine heart and yeast mitochondria (Esposti et al., 1983; Clejan & Beattie, 1983), and by the bovine cytochrome *c* oxidase (Casey et al., 1980) is inhibited by the carboxyl modifying reagent DCCD.[1] In the mammalian ATP synthase complex, DCCD reacts at two sites, a proteolipid in the membrane sector, F_0, and the β subunit of the catalytic segment, F_1. However, at 0 °C and low concentrations of DCCD, only the proteolipid, which is involved in transmembrane proton translocation, is modified (Kiehl & Hatefi, 1980). In the ATP synthase complex (F_1–F_0), modification of F_0 by DCCD perforce inhibits the catalytic function of F_1, even though the F_1 moiety exhibits full activity when separated from the DCCD-modified F_0. In cytochrome *c* oxidase the primary target for DCCD is subunit III (Casey et al., 1980) while the electron carriers of the enzyme (hemes *a* and a_3, Cu_a, and Cu_{a3}) appear to be contained in subunits I and II (Capaldi et al., 1983). Hence, it can be shown under appropriate conditions that modification of cytochrome *c* oxidase by DCCD inhibits primarily the vectorial proton translocation by the enzyme complex rather than the scalar electron-transfer reaction (Casey et al., 1980). A similar differential effect of DCCD has also been observed in the case of complex III (Clejan & Beattie, 1983; Esposti et al., 1983).

Another mitochondrial energy-transducing enzyme that is inhibited by DCCD is the energy-linked nicotinamidenucleotide transhydrogenase (TH) (Phelps & Hatefi, 1981; Pennington & Fisher, 1981). The former authors showed that SMP-bound TH is protected against DCCD inhibition by NAD(H) and analogues, thus suggesting that the binding site of DCCD might be close to that of NAD(H). By contrast, Pennington & Fisher (1981) could not demonstrate protection by NAD(H) and presented data indicating that DCCD inhibits proton translocation by TH much more than hydride ion transfer from NADPH to AcPyAD. Thus, they concluded that DCCD binds outside the active site and modifies a residue in the proton channel of the enzyme. More recently, we have further confirmed our earlier findings and shown (a) that NAD(H) and analogues, including the NAD(H) competitive inhibitors 5′-AMP and 5′-ADP, protect the purified and the membrane-bound TH against inhibition by DCCD while 2′-AMP and 3′-AMP, which are competitive inhibitors of NADP(H), do not protect and (b) that, unlike the native TH, the DCCD-modified enzyme does not bind to NAD–agarose. (Phelps & Hatefi, 1984).

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; AcPyAD, 3-acetylpyridine adenine dinucleotide; AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; F_1, mitochondrial F_1-ATPase; TH, nicotinamidenucleotide transhydrogenase or the purified transhydrogenase enzyme; SMP, submitochondrial particles.

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