New insight into the pyruvate decarboxylase-catalysed formation of lactaldehyde from H–D exchange experiments: a 'water proof' active site

Mario Lobell and David H. G. Crout *

*Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK*

Pyruvate decarboxylase from *Saccharomyces cerevisiae* catalyses the formation of lactaldehyde from sodium glyoxylate and acetaldehyde. By using deuteriated sodium glyoxylate (sodium [2-^{2}H]glyoxylate monohydrate) as a substrate it was verified that the lactaldehyde formed retains the deuterium atom. The implications of the observed result for the enzyme mechanism are discussed in the light of conclusions derived from recent molecular modelling studies.

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is a thiamin diphosphate (TDP)-dependent enzyme that catalyses decarboxylation of pyruvate to acetaldehyde and carbon dioxide. Its mechanism of action has been extensively studied,[1-8] particularly in relation to the role of the cofactor TDP. The availability of an X-ray crystal structure[9] has made possible a detailed analysis of the mechanism of action of TDP both with respect to decarboxylation of pyruvate (Scheme 1, 1→8) and to the 'anomalous' reaction whereby the carbanion-enamine intermediate attacks another carbonyl compound (*e.g.* benzaldehyde) to generate a new carbon–carbon bond[10-16] (Scheme 1, 1→5, 9→12).
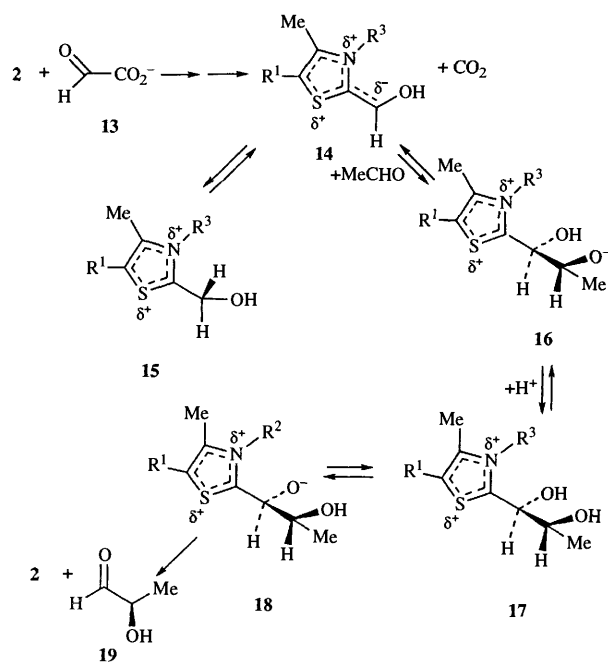
Trace amounts of intermediate 6 have been isolated by radio paper chromatography from incubation mixtures of PDC and ^{14}C-labelled pyruvate.[17] However, intermediate 6 cannot be isolated in substantial yield and therefore its absolute configuration is not known. Modelling studies[18] strongly suggest that it has the *R*-configuration shown. If the stereochemical course of the TDP reaction is to be fully described, it will be necessary to confirm this assignment experimentally. A method whereby this might be carried out is based on the following considerations.

Glyoxylate 13 is an inhibitor of yeast PDC.[19,20] The intermediate 14 (Scheme 2) analogous to the intermediate 5 formed from pyruvate (Scheme 1) is produced and protonated to give the hydroxymethyl analogue 15 of intermediate 6. However, unlike intermediate 6, intermediate 15 does not undergo retro-aldol cleavage to formaldehyde and the TDP anion 2, and it should be possible to isolate it from the incubation mixture following the procedure[21] for its isolation from pyruvate oxidase from pig heart muscle. However, intermediate 14 can add to acetaldehyde, if present in high enough concentration, to give, eventually, lactaldehyde 19 *via* the sequence 13→14, 16→19 (Scheme 2).[22] The reaction is slow and yields only 1–2% of lactaldehyde.
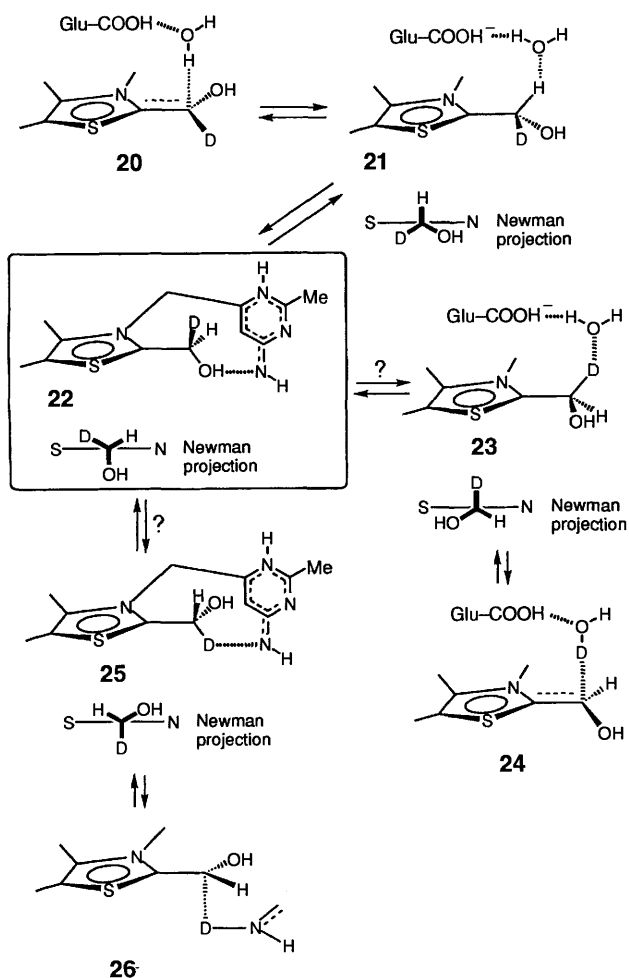
The C-α carbon atom of the 2-hydroxymethyl intermediate 15 is achiral. However, it can be made chiral if deuteriated glyoxylate ([2-^{2}H]glyoxylate) is used. In principle, this could be used as a basis for determining the stereochemistry of formation of intermediate 15. However, an important preliminary to such an investigation is to confirm that the stereochemical integrity of this intermediate is maintained during the catalytic process. Molecular modelling studies suggest that the carbanion-enamine intermediate 20 (Scheme 3) is protonated by Glu 477 aided by a bridging water molecule. The 2-hydroxymethyl-TDP intermediate 21, with the *R*-configuration, is formed. Energy minimisation of intermediate 21 brings the hydroxy group into close contact with the deprotonated 4'-imino group (intermediate 22). The 4'-imino group can form a hydrogen bond to the

Scheme 1
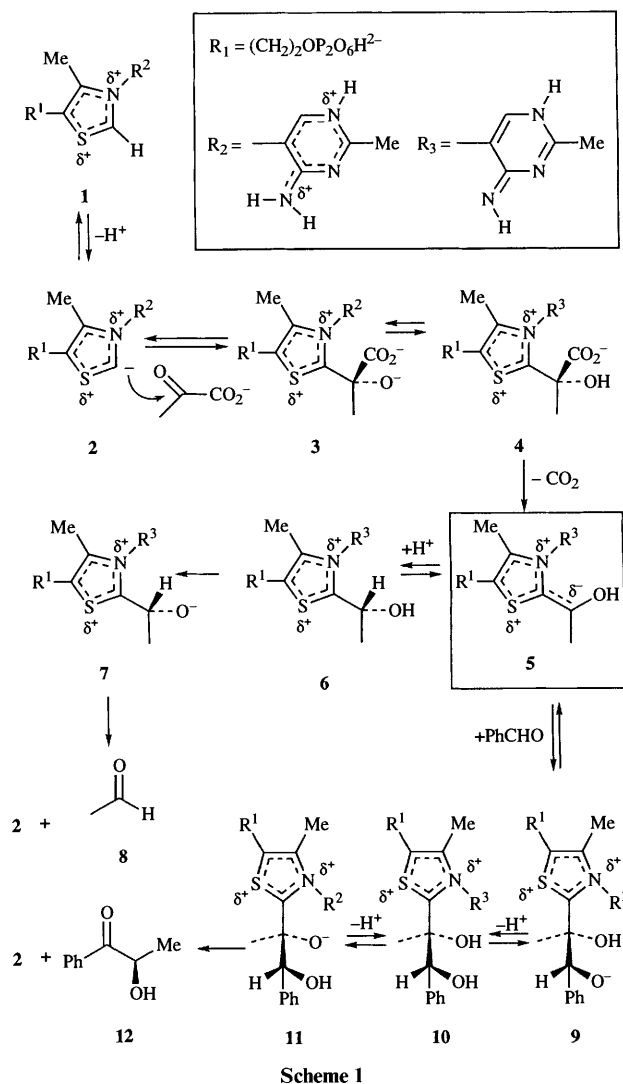
hydroxy proton, but the proton either is not abstracted, or is abstracted to give a stable alkoxide species that does not undergo retro-aldol cleavage. Reversal of this process leads back to the carbanion-enamine intermediate 20, conserving the pattern of isotopic labelling. Addition of intermediate 20 to acetaldehyde would yield deuteriated lactaldehyde ([^{2}H]lactaldehyde). However, it was important to consider the possibility that there might be deuterium–protium exchange which would
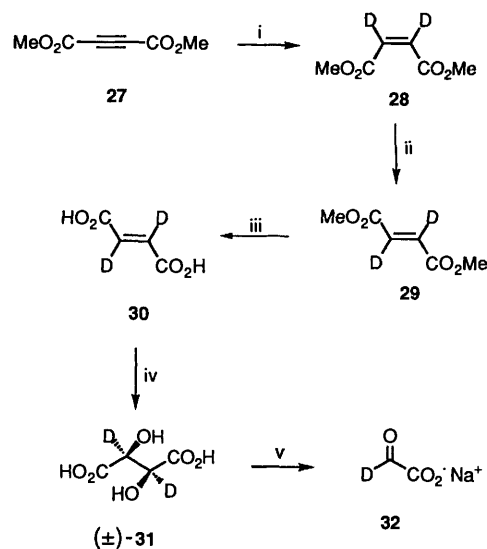
Scheme 2

Scheme 3

Newman projection (labels: 20, 21, 22, 23, 24, 25, 26)

Scheme 4 *Reagents:* i, D_2–Lindlar catalyst; ii, I_2; iii, HNO_3; iv, OsO_4–$NaClO_4$; v, HIO_4

24 would not be expected to form lactaldehyde as the hydroxy group points away from the basic 4'-imino group which is the species that abstracts the proton in the retro-aldol reaction leading to product release. Second, intermediate **22** might rearrange to **25** followed by proton transfer to the 4'-imino group. For this process to lead to overall H–D exchange, intermediate **26** would need to undergo D–H exchange. However, molecular modelling studies indicate that the 4'-imino group of the intermediate **27** is completely inaccessible to solvent and thus not susceptible to H–D exchange. If H–D exchange were found to occur, this would indicate a marked conformational change in the enzyme during the catalytic process.

In order to verify whether any deuterium–protium exchange could be observed, deuteriated sodium glyoxylate **32** (sodium [2-^{2}H]glyoxylate)[23] was synthesised (Scheme 4) and was used as substrate in the PDC-catalysed formation of lactaldehyde. In a complementary experiment deuterium oxide was used as solvent for the PDC-catalysed formation of lactaldehyde from sodium glyoxylate and acetaldehyde. The lactaldehyde produced in each experiment was derivatised with 2,4-dinitrophenylhydrazine and the resulting derivatives were isolated and analysed by ^{1}H NMR.

The deuterium content of the 2,4-dinitrophenylhydrazone derivative **33** of the sodium [2-^{2}H]glyoxylate and the derivative **34** of the product lactaldehyde were identical. The lactaldehyde derivative from the complementary experiment with deuterium oxide did not contain deuterium.

As expected from the results of the molecular modelling studies, this experimental verification confirmed that no deuterium–hydrogen exchange takes place during the PDC-catalysed formation of lactaldehyde from sodium [^{2}H]glyoxylate in H_2O or from sodium glyoxylate in D_2O.

This finding lends further support to the suggestion[24] that the active site in PDC is closed during critical proton transfer steps in PDC-catalysed reactions. This is necessary in order to maintain the 4'-amino group in the correct state for catalysis. If molecules of water had free access to the active site during catalysis, buffering of groups involved in proton transfer reactions would occur and catalysis would be drastically reduced or eliminated entirely. The putative water molecule associated with Glu 477 acts as an integral part of the protein structure. This molecule of water also appears to be isolated from the hydration sphere of the enzyme. Proton transfer to and from this water molecule during pyruvate decarboxylation is not coupled with equilibration with external water. Harris and Washabaugh[25] recently studied the distribution of tritium

lead to lactaldehyde containing no deuterium or with a reduced deuterium content. This might occur through the operation of one of two possible mechanisms. First, intermediate **22** might rearrange to intermediate **23** (Scheme 3). This might undergo proton transfer *via* proton exchange, to the water molecule bridge to Glu 477 (**23**, Scheme 3). However, carbanion-enamine

derived from enzyme-bound C(2)-tritiated TDP during the reaction of pyruvate to form acetaldehyde. On single turnover conditions they found that 43% of the tritium had been transferred to the acetaldehyde formed and 54% to water. This result demonstrates that the initial deprotonation of TDP (1→2, Scheme 1) and subsequent protonation of the carbanion-enamine intermediate (5→6, Scheme 1) are connected in some way. The following proposed pathway might provide an explanation. Thus the initially deprotonated residue Glu 477 abstracts a proton from a water molecule to generate a hydroxide ion. This hydroxide ion then abstracts the C(2)-bound tritium ion (Fig. 1) generating the tritiated water molecule which subsequently transfers its tritium ion (or proton) to the carbanion-enamine intermediate 5 assisted by Glu 477. It could be shown by molecular modelling that a hydroxide ion positioned close to the C(2)–H group of enzyme bound TDP easily minimises into a docking position (Fig. 1) favourable for abstraction of the C(2)-bound proton. The hydroxide ion is firmly held in place by three hydrogen bonds to Asp 28, the 4'-amino and the C(2)–H group.

Experimental

Dimethyl acetylenedicarboxylate, deuterium gas, Lindlar catalyst (Pd on $CaCO_3$, poisoned with Pb), osmium tetraoxide, periodic acid, deuterium oxide and 2,4-dinitrophenylhydrazine (70% weight content) were purchased from Aldrich. Sodium glyoxylate monohydrate was purchased from Fluka. Thiamin diphosphate (TDP) was purchased from Sigma. Pyruvate decarboxylase (PDC) from *Saccharomyces cerevisiae* was purchased from Sigma as a suspension in 3.2 mol dm^{-3} ammonium sulfate, pH 6.5, stabilised with 5% glycerol, 5 mmol dm^{-3} potassium phosphate, 1 mmol dm^{-3} magnesium acetate, 0.5 mmol dm^{-3} ethylenediamine tetraacetic acid (EDTA) and 25 mmol dm^{-3} TDP. The suspension contained 15 mg cm^{-3} protein with a specific activity of 5.2 U mg^{-1} protein. NMR spectra were obtained at 250 MHz using a Bruker AFC250 spectrometer. Mass spectra were determined using a Kratos MS80 spectrometer. NMR coupling constants (J) are quoted in Hz. Melting points are uncorrected. Sodium [2-^{2}H]glyoxylate
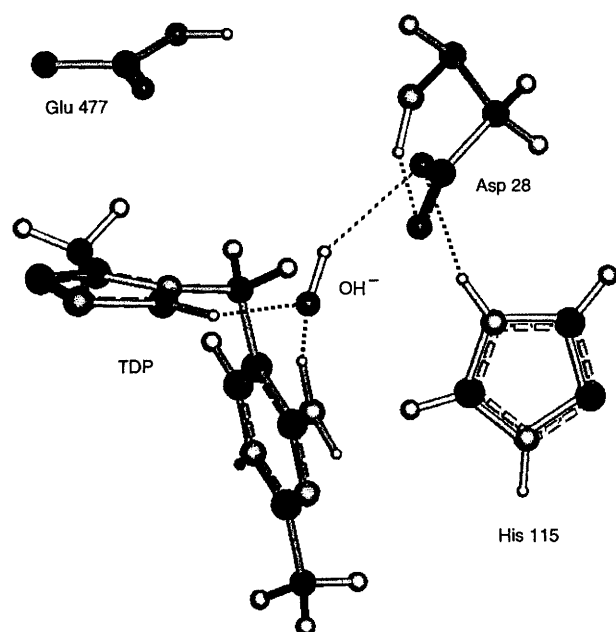

Fig. 1 Ball and stick representation of the energy minimised active site of pyruvate decarboxylase including a hydroxide ion bound near to the C(2)–H group of TDP. The hydroxide ion is firmly held in place by three hydrogen bonds to Asp 28, the 4'-amino and C(2)–H group. Calculated hydrogen bonds are drawn as dashed lines.

monohydrate (as 32) was synthesised by a reaction sequence developed by H. Weber.[23] The procedures reported by Weber have been slightly modified to increase yields.

Molecular modelling

All calculations were performed on a Silicon Graphics Iris Indigo work station. QUANTA (ver. 4.1, Molecular Simulations Inc., Burlington, MA, USA) was used for construction and analysis of all modelled structures. Energy minimisations were performed using CHARMm (subprogram used within QUANTA) and the Adopted Basis Newton–Raphson Minimisation Algorithm. Electrostatic interactions were calculated by applying a distance-dependent dielectric term (value of dielectric constant: 4). Nonbonded interactions were calculated with a cutoff distance of 12 Å. The nonbonded atom list was updated every 25 minimisation steps. A switching smoothing function was applied to the van der Waals and electrostatic terms between 8 and 10 Å distance.

Each yeast PDC monomer contains a single polypeptide chain. Two monomers are tightly associated to form a dimer with approximate 2-fold symmetry. A 2-fold rotation of the dimer about one axis generates another dimer, which contacts the first to form a complete PDC tetramer. The TDPs are situated at the interface between monomers within each tightly associated dimer, with two sites per dimer and four per tetramer. The computer model of PDC contains one dimer, which is represented by all carbon and heteroatoms of the two polypeptide chains (A- and B-chain) and the two bound TDP molecules. Predefined residue topology files were used by the HBUILD function of CHARMm to add all hydrogen atoms (polar and non polar). In general, aspartic acid and glutamic acid residues have not been protonated; the only exception is Glu 477. Protonation of histidine residues was decided according to hydrogen bonding pattern and solvent accessibility: His 97 (uncharged, proton on N3), His 481 (uncharged, proton on N1), His 92, 114, 115, 126, 225, 310, 313, 495, 510 (charged, both N protonated). All arginine and lysine residues were protonated.

The 4'-amino group was held in the plane of the pyrimidine ring by applying an additional dihedral constraint [harmonic force constant: 50 kcal mol^{-1} (209.2 kJ mol^{-1})].

During energy minimisation all enzyme atoms with the exception of some few residues were fixed in their positions as found in the crystal structure. The residues allowed to relax were TDP (or a corresponding derivative) excluding the diphoshate group, Glu 477(A), Asp 28(B), His 115(B) and the docking hydroxide ion.

Dimethyl [2,3-^{2}H$_2$]fumarate 29

Using a hydrogenator dimethyl acetylenedicarboxylate (9.84 cm^3, 80 mmol) was dissolved in dry ethyl acetate (90 cm^3) and deuteriated for 43 h at room temperature in the presence of Lindlar catalyst (2.2 g) and under a D_2-pressure of about 1 atm. The mixture was filtered to remove the catalyst and the ethyl acetate evaporated under reduced pressure. Vacuum distillation yielded dimethyl [2,3-^{2}H$_2$]maleate 28 (9.16 g, 78%) (bp 132–138 °C at 80 torr). An iodine crystal was added and the mixture was boiled under reflux (bp 190–200 °C) for 4 h. The mixture was allowed to cool and the product was recrystallised (diethyl ether). The dimethyl [2,3-^{2}H$_2$]fumarate 29 (4.97 g, 43%) was filtered off and the filtrate was evaporated to recover residual dimethyl [2,3-^{2}H$_2$]maleate 28 for an additional iodine catalysed isomerisation, which yielded further dimethyl [2,3-^{2}H$_2$]fumarate 29 (0.60 g, 5%), mp 102–103 °C (from diethyl ether) (lit.,[23] 103 °C, lit.,[26] 98–101 °C, lit.,[27] 98–100 °C); δ_H(CDCl$_3$; SiMe$_4$) 3.78 (6 H, s, 2 × Me), 6.82 (0.2 H, m, CH=CD); δ_C(CDCl$_3$; SiMe$_4$) 52.2 (Me), 132.9 (t, CD=), 133.2 (CH=), 165.2 (C=O); m/z (EI, 70 eV) 146 (M^+, dimethyl [2,3-^{2}H$_2$]fumarate, 78%), 145 (M^+, dimethyl [2-^{2}H]fumarate, 19%) and 144 (M^+, dimethyl fumarate, 3%).

[2,3-²H₂]Fumaric acid 30

Dimethyl [2,3-²H₂]fumarate 29 (4.45 g, 30.45 mmol) was boiled under reflux in nitric acid (1 mol dm⁻³; 50 cm³). Methanol (3 cm³) was added to prevent the volatile starting material subliming into the reflux condenser. After 24 h the solution was concentrated under reduced pressure. The crystallised product was filtered off (using a filter material inert to conc. HNO₃) and recrystallised from water yielding [2,3-²H₂]fumaric acid 30 (2.694 g, 75%), mp 290–292 °C (from water) (lit.,[23] 292 °C, lit.,[27] 296–300 °C); δ_C[(CD₃)₂SO; SiMe₄] 134.0 (t, CD=), 134.1 (CH=), 166.0 (C=O).

[2,3-²H₂]Tartaric acid 31

Osmium tetraoxide (0.1–0.2 g) was added to an aqueous solution (30 cm³) containing [2,3-²H₂]fumaric acid 30 (2.065 g, 17.5 mmol), sodium carbonate (1.2 g, 11.3 mmol) and sodium perchlorate (2.5 g, 23.5 mmol). The mixture was kept under a nitrogen atmosphere and stirred at 40–45 °C over night. The aqueous solution was washed with five portions of diethyl ether (20 cm³). The aqueous phase was acidified (HCl), saturated with sodium chloride and continuously extracted with ethyl acetate (250 cm³) for 6 days. Evaporation of the ethyl acetate under reduced pressure yielded the [2,3-²H₂]tartaric acid 31 (1.193 g, 45%), mp 205–207 °C (lit.,[23] 207 °C); δ_C[(CD₃)₂SO; SiMe₄] 71.9 (t, CDOH), 72.2 (CHOH), 173.2 (C=O).

Sodium [2-²H]glyoxylate monohydrate 32

A solution of [2,3-²H₂]tartaric acid 31 (1.019 g, 6.7 mmol) in water (6.7 cm³) was cooled in an ice bath, a small piece of ice was added and a solution of periodic acid (1.89 g, 8.2 mmol) in water (5 cm³) was slowly added dropwise. After each 1.5 cm³ portion a new piece of ice was added to the mixture. After the addition was complete the ice bath was removed and stirring continued for 20 min. The solution was filtered to remove insoluble ester impurities followed by continuous extraction with diethyl ether (250 cm³) for 3 days. The ether solution was concentrated under reduced pressure to between 5 and 10 cm³, transferred into a 25 cm³ flask, and the ether was removed under reduced pressure. Just enough distilled water (1.5 cm³) was added to bring the residue into solution and NaOH (10 mol dm⁻³; 0.75 cm³) was added. Upon addition of acetone (2.5 cm³) the colourless product crystallised from the solution. Crystallisation was complete after 2 h at 4 °C. Filtration yielded sodium [2-²H]glyoxylate monohydrate (as 32) (622 mg, 40%); δ_H(D₂O; Me₃Si(CH₂)₂CO₂⁻Na⁺) 5.09 [1 H, s, CH(OH)₂], 8.46 (0.3 H, s, CH=O); δ_C(D₂O; Me₃Si(CH₂)₂CO₂⁻Na⁺) 90.5 (t, J 25.1, CDOH), 90.8 (CHOH), 176.1 (O=C–CO₂⁻), 179.6 [(HO)₂C–CO₂⁻]. The correct identity of the product was confirmed by characterisation of the 2,4-dinitrophenylhydrazone derivative 33 by ¹H NMR (Fig. 2). Apart from its incomplete deuteriation, 33 is a Z/E mixture with regard to the C=N double bond. Accordingly, the measured melting point range of 149–154 °C significantly differs from the mp for the pure Z isomer (lit.,[28] 182.5–183.5 °C) and E isomer (lit.,[28] 205.5 °C) of the non-deuteriated derivative.

PDC-catalysed formation of lactaldehyde 19 from sodium glyoxylate monohydrate and acetaldehyde in deuterium oxide and isolation as lactaldehyde 2,4-dinitrophenylhydrazone

Sodium glyoxylate monohydrate (114 mg, 1 mmol), sodium hydroxide (112 mg, 2.8 mmol), citric acid hydrate (210 mg, 1 mmol), a small crystal of TDP, a small crystal of magnesium chloride and acetaldehyde (0.336 cm³, 6 mmol) were mixed with 8.7 cm³ of deuterium oxide. PDC suspension (0.6 cm³, 47 U) was added to the resulting solution and the reaction mixture was stirred in a stoppered 25 cm³ flask for 2 days at about 25 °C. To remove some of the excess acetaldehyde the flask was then connected to a rotary evaporator and evaporated under reduced pressure (ca. 200 torr) at about 25 °C bath temperature
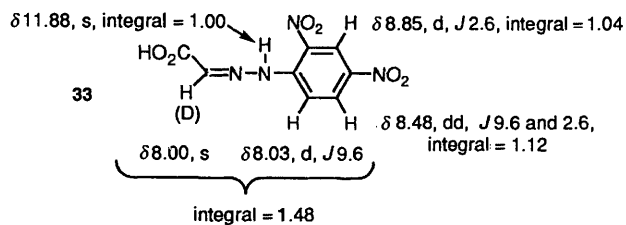
Fig. 2 NMR assignments for the 2,4-dinitrophenylhydrazone 33 derived from sodium [2-²H]glyoxylate monohydrate 32

for about 30 min. The aldehydes in the residual mixture were transformed into their 2,4-dinitrophenylhydrazones by the following procedure: concentrated sulfuric acid (24 cm³) and then distilled water (24 cm³) were added to 2,4-dinitrophenylhydrazine (1.70 g, 6 mmol), the mixture was swirled until the reagent was dissolved and the solution diluted with further water (450 cm³). The aldehyde solution was added and the mixture was stirred for 20 min. The resulting orange precipitate was filtered, washed with distilled water (3 cm³) and dried under reduced pressure overnight. The lactaldehyde derivative was isolated from the mixture of 2,4-dinitrophenylhydrazones (289 mg) by chromatography on silica gel with ethyl acetate–light petroleum (bp 40–60 °C) (1:1) as the eluent yielding pure lactaldehyde 2,4-dinitrophenylhydrazone (6 mg, 2%), R_f 0.5, mp 153–156 °C (lit.,[23] 155–156 °C, lit.,[29,30] 155–158 °C); δ_H(CDCl₃; SiMe₄) 1.47 (3 H, d, J 6.7, Me), 4.63 (1 H, m, CHOH), 7.57 (1 H, dd, J 3.8 and 0.9, NCH), 7.91 (1 H, d, J 9.6, 6-H-Ph), 8.32 (1 H, dd, J 9.6 and 2.6, 5-H-Ph), 9.11 (1 H, d, J 2.6, 3-H-Ph) and 11.09 (1 H, s, NH).

PDC-catalysed formation of [1-²H]lactaldehyde from sodium [2-²H]glyoxylate monohydrate and acetaldehyde in water and isolation in form of the [1-²H]lactaldehyde 2,4-dinitrophenylhydrazone 34

The procedure was identical to the one described above. Starting from sodium [2-²H]glyoxylate monohydrate (as 32) (115 mg, 1 mmol) and using water instead of deuterium oxide as reaction solvent the procedure yielded [1-²H]lactaldehyde 2,4-dinitrophenylhydrazone 34 (3 mg, 1%); δ_H(CDCl₃; SiMe₄) 1.47 (3 H. d, J 6.7, Me), 4.63 (1 H, m, CHOH), 7.57 (0.4 H, dd, J 3.8 and 0.9, NCH), 7.91 (1 H, d, J 9.6, 6-H-Ph), 8.32 (1 H, dd, J 9.6 and 2.6, 5-H-Ph), 9.11 (1 H, d, J 2.6, 3-H-Ph) and 11.09 (1 H, s, NH).

Determination of isotopic compositions

The derivatisation of aldehydes with 2,4-dinitrophenylhydrazine is accompanied by some exchange of formyl protons with solvent protons. Therefore, the deuteriation grade of [1-²H]lactaldehyde drops during derivatisation. In order to analyse if the deuteriation grade has changed during the PDC-catalysed formation of [1-²H]lactaldehyde, it was necessary to compare the deuteriation grades of the 2,4-dinitrophenylhydrazones of the sodium [2-²H]glyoxylate monohydrate used and of the product [1-²H]lactaldehyde 2,4-dinitrophenylhydrazone 34.

Sodium [2-²H]glyoxylate monohydrate (58 mg, 0.5 mmol) was derivatised with 2,4-dinitrophenylhydrazine (357 mg, 1.26 mmol) according to the procedure described above. The ¹H NMR spectrum of the 2,4-dinitrophenylhydrazone 33 in (CD₃)₂SO showed four signals (Fig. 2).

On average, integration yielded $(1.00 + 1.05 + 1.12)/3 = 1.05$ arbitrary units per proton, which means that $(1.48 - 1.05) = 0.43$ of the integral for the superimposed signal can be assigned to the partly deuteriated position. Therefore, the hydrogen content in this position is $(0.43/1.05) \times 100 = 41\%$.

The ¹H NMR spectrum of the [1-²H]lactaldehyde 2,4-dinitrophenylhydrazone 34 in CDCl₃ showed seven signals (Fig. 3). Integration of the signal attributable to the methyl

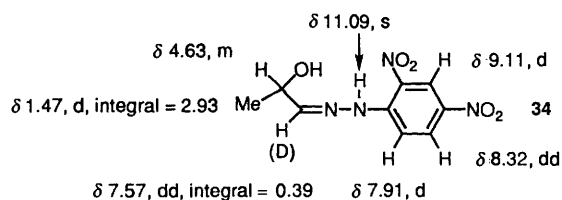**Fig. 3** NMR assignments for the 2,4-dinitrophenylhydrazone **34** derived from [1-²H]lactaldehyde

group yielded 2.93/3 = 0.98 arbitrary units per proton. Integration of the signal from the partly deuteriated position gave 0.39 arbitrary units. Accordingly, the hydrogen content in this position was (0.39/0.98) × 100 = 40%.

The ¹H NMR spectrum of the 2,4-dinitrophenylhydrazone of lactaldehyde produced in D_2O was analysed in the same way. Integration of the signal attributable to the methyl group gave 5.65/3 = 1.88 arbitrary units per proton. Integration of the signal from the HC=N group yielded 1.99 arbitrary units. Accordingly, no deuterium/hydrogen exchange was observed.

Acknowledgements

References

1 A. Schellenberger, *Angew. Chem., Int. Ed. Engl.*, 1967, **6**, 1024.
2 G. Hübner, H. Neef, G. Fischer and A. Schellenberger, *Z. Chem.*, 1975, **15**, 221.
3 R. Kluger, *Chem. Rev.*, 1987, **87**, 863.
4 A. Schellenberger, *Chem. Ber.*, 1990, **123**, 1489.
5 F. J. Alvarez, J. Ermer, G. Hübner, A. Schellenberger and R. L. Schowen, *J. Am. Chem. Soc.*, 1991, **113**, 8402.
6 J. Ermer, A. Schellenberger and G. Hübner, *FEBS Lett.*, 1992, **299**, 163.
7 E. J. Crane, J. A. Vaccaro and M. W. Washabaugh, *J. Am. Chem. Soc.*, 1993, **115**, 8912.
8 T. H. Harris and M. W. Washabaugh, *Biochemistry*, 1995, **34**, 14001.
9 F. Dyda, W. Furey, S. Swaminathan, M. Sax, B. Farrenkopf and F. Jordan, *Biochemistry*, 1993, **32**, 6165.
10 C. Neuberg and J. Hirsch, *Biochem. Z.*, 1921, **115**, 282.
11 S. Bringer-Meyer and H. Sahm, *Biocatalysis*, 1988, **1**, 321.
12 D. H. G. Crout, H. Dalton, D. W. Hutchinson and M. Miyagoshi, *J. Chem. Soc., Perkin Trans. 1*, 1991, 1329.
13 R. Cardillo, S. Servi and C. Tinti, *Appl. Microbiol. Biotechnol.*, 1991, **36**, 300.
14 J. T. Stivers and M. W. Washabaugh, *Biochemistry*, 1993, **32**, 13472.
15 V. Kren, D. H. G. Crout, H. Dalton, D. W. Hutchinson, W. König, M. M. Turner, G. Dean and N. Thomson, *J. Chem. Soc., Chem. Commun.*, 1993, 341.
16 S. Bornemann, D. H. G. Crout, H. Dalton, V. Kren, M. Lobell, G. Dean, N. Thomson and M. M. Turner, *J. Chem. Soc., Perkin Trans. 1*, 1996, 425.
17 H. Holzer and K. Beaucamp, *Biochim. Biophys. Acta*, 1961, **46**, 225.
18 M. Lobell and D. H. G. Crout, *J. Am. Chem. Soc.*, 1996, **118**, 1867.
19 H. Uhlemann and A. Schellenberger, *FEBS Lett.*, 1976, **63**, 37.
20 H. Holzer and H. W. Goedde, *Biochem. Z.*, 1957, **329**, 192.
21 G. Kohlhaw, B. Deus and H. Holzer, *J. Biol. Chem.*, 1965, **240**, 2135.
22 S. Bornemann, D. H. G. Crout, H. Dalton, D. W. Hutchinson, G. Dean, N. Thomson and M. M. Turner, *J. Chem. Soc., Perkin Trans. 1*, 1993, 309.
23 H. Weber, Ph.D. Thesis, ETH Zurich, 1965.
24 F. J. Alvarez, J. Ermer, G. Hübner, A. Schellenberger and R. L. Schowen, *J. Am. Chem. Soc.*, 1995, **117**, 1678.
25 T. H. Harris and M. W. Washabaugh, *Biochemistry*, 1995, **34**, 13994.
26 J. R. Merchant and J. R. Patell, *J. Chem. Soc. (C)*, 1969, 1544.
27 K. M. Lee, K. Ramalingam, J. K. Son and R. W. Woodard, *J. Org. Chem.*, 1988, **54**, 3195.
28 H. Katsuki, C. Tanegashima, M. Tokushige and S. Tanaka, *Bull. Chem. Soc. Jpn.*, 1972, **45**, 813.
29 C. Zioudrou and P. Chrysochou, *Tetrahedron*, 1977, **33**, 2103.
30 E. Huff and H. Rudney, *J. Biol. Chem.*, 1959, **234**, 1060.