Mudd, S. H., Ebert, M. H., & Scriver, C. R. (1980) *Metabolism 29*, 707–720.
Olsen, J., Wu, Y. S., Borchardt, R. T., & Schowen, R. L. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 127–133, Elsevier/North-Holland, New York.
Parks, L. W., & Schlenk, F. (1958) *J. Biol. Chem. 230*, 295–305.
Savige, W. E., & Fontana, A. (1977) *Methods Enzymol. 47E*, 453–459.
Scartazzini, R., & Mislow, K. (1967) *Tetrahedron Lett. 28*, 2719–2722.
Schlenk, R., & Zydek-Cwick, C. R. (1969) *Arch. Biochem. Biophys. 134*, 414–422.
Sharpless, T. K., & Wolfenden, R. (1967) *Methods Enzymol. 12A*, 126–131.
Smith, J. D. (1976) *Biochem. Biophys. Res. Commun. 73*, 7–12.
Stolowitz, M. L., & Minch, J. J. (1981) *J. Am. Chem. Soc. 103*, 6015–6019.
Streitwieser, A. (1962) *Solvolytic Displacement Reactions*, p 13, McGraw-Hill, New York.
Swain, C. G., & Taylor, L. J. (1962) *J. Am. Chem. Soc. 84*, 2456–2457.
Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds. (1979) *Transmethylation*, Elsevier/North-Holland, New York.
Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds. (1981) *Biochemistry of S-Adenosylmethionine and Related Compounds*, Macmillan Press, London.
Yagi, K. (1971) *Methods Enzymol. 17B*, 608–622.
Zappia, V., Galletti, P., Olivia, A., & DeSantis, A. (1977) *Anal. Biochem. 79*, 535–543.
Zappia, V., Carteni-Farina, M., & Porcelli, M. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 95–104, Elsevier/North-Holland, New York.

Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System: Stereospecificity of Proton Transfer in the Phosphorylation of Enzyme I from (*Z*)-Phosphoenolbutyrate†

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ABSTRACT: The stereochemistry of the proton transfer in the reaction of phosphoenolbutyrate with enzyme I has been established. During the reaction of the pure *Z* isomer of this analogue of phosphoenolpyruvate with enzyme I, to yield phosphoenzyme I and 2-oxobutyrate, the substrate is protonated at C-3 from the 2*re*,3*si* face. This stereospecificity was established for the transfer of a proton to (*Z*)-phospho[3-D]enolbutyrate and for the transfer of a deuteron to (*Z*)-phospho[3-H]enolbutyrate. The *E* isomer of phosphoenolbutyrate is not a substrate for enzyme I. Accordingly, the reaction of phosphoenzyme I with 2-oxobutyrate yields exclusively the *Z* isomer of phosphoenolbutyrate, and only the *pro-S* proton at C-3 of 2-oxobutyrate is abstracted. A kinetic H/D isotope effect of 6.8 in this reaction demonstrates the rate-limiting nature of the proton-transfer step. The stereochemical analysis of 2-oxo[3(*R*)-H,D]butyrate and of 2-oxo-[3(*S*)-H,D]butyrate was carried out by using the pyruvate kinase catalyzed enolization of this compound. This enzymatic enolization, with phosphate as a cofactor, is rapid at neutral pH and is a highly stereospecific reaction: only the *pro-R* proton at C-3 of 2-oxobutyrate is exchanged with solvent. This reaction was also used to generate the pure 3*R* and 3*S* enantiomers of 2-oxo[3-H,D]butyrate. The degree of protonation/deuteration at C-3 of 2-oxobutyrate was detected from the fine structure of the methyl proton nuclear magnetic resonance signal.

Enzyme I, a component of the bacterial PEP[1]-dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phosphocarrier protein HPr. This is the first step in a process that ultimately leads to the phosphorylation and concomitant transport of PTS sugars into the bacterial cell [for recent reviews, see Hays (1978) and Robillard (1982)]. The enzyme I catalyzed reaction proceeds via a phosphoenzyme intermediate (Stein et al., 1974; Waygood & Steeves, 1980; Saier et al., 1980; Hoving et al., 1981). After phosphorylation of enzyme I by PEP, ketopyruvate is the product species that dissociates from the phosphorylated enzyme. It is still unclear whether the enolate of pyruvate is an enzyme-bound intermediate during the reaction or if phosphoryl-group transfer and protonation take place in a concerted fashion. It has been clearly demonstrated, however, that the proton transfer does take place on the enzyme and that the base on enzyme I that is responsible for this proton transfer is not in protonic equilibrium with the solvent during the course of a reaction (Hoving et al., 1981). Since the protonation of the enol substrate is carried out by the enzyme, the reaction may be expected to take place stereospecifically. Thus, information on the extent to which the proton transfer

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[1] Abbreviations: PEP, phosphoenolpyruvate; PEB, phosphoenolbutyrate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DTT, dithiothreitol; NMR, nuclear magnetic resonance; 2-OB, 2-oxobutyrate; D, ^{2}H; T, ^{3}H.

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is stereospecific, together with kinetic data relevant to this reaction step, may provide valuable clues to the reaction mechanism. Knowledge of the stereochemical course of the protonation step will be of value in determining the spatial arrangement of catalytic groups on the enzyme.

Stereospecificity of proton transfer to the C-3 atom of PEP can only be determined if the reaction product, i.e., pyruvate, is chiral. This can be achieved by making a specific isotopic substitution for one of the vinyl protons of PEP and using yet another isotope in the enzymatic protonation step. Rose (1970) successfully applied this isotopic substitution method to the enzyme pyruvate kinase and, using the elegant method developed by Cornforth (1969) and Arigoni and co-workers (Lüthy et al., 1969) to determine the chirality of the resulting pyruvate methyl group, demonstrated that this enzyme protonates PEP with high specificity from the 2*si* face.[2] Other substitutions on PEP are also possible, providing the enzyme under study can use the PEP analogue as a substrate. Pure *E* and *Z* isomers[2] of various PEP analogues have been synthesized and tested as pseudosubstrates for PEP-utilizing enzymes (Bondinell & Sprinson, 1970; Stubbe & Kenyon, 1971, 1972; Blumberg & Stubbe, 1975; Adlersberg et al., 1977; Duffy et al., 1982).

In this paper, studies will be presented in which the known stereospecificity of pyruvate kinase was used to elucidate the stereochemistry of PEB protonation in the reaction with enzyme I. Experiments monitoring the stereochemical course of H and D transfer will be complemented with studies on the kinetic H/D isotope effect. In addition to the reaction of PEB with enzyme I, the stereochemistry of the reverse reaction between 2-oxobutyrate and phosphoenzyme I was investigated. Data will also be presented to show that the enzyme I catalyzed transphosphorylation reaction from PEP to 2-oxobutyrate can be used to generate the pure *Z* isomer of PEB.

Materials and Methods

Chemicals. Phosphoenolpyruvate monopotassium salt, pyruvate sodium salt, 3-fluoropyruvate sodium salt, and 2-oxobutyrate sodium salt were purchased from Sigma Chemical Co. and were used without further purification. [1-^{14}C]-Phosphoenolpyruvate monocyclohexylammonium salt (sp act. 12 mCi/mmol) was purchased from Amersham.

Enzymes. Pyruvate kinase (from rabbit muscle) and lactate dehydrogenase (from pig muscle) were purchased from Boehringer. Enzyme I was purified from *Escherichia coli* P650 as described previously (Robillard et al., 1979; Brouwer et al., 1982). Enzyme I concentrations were determined as described by Brouwer et al. (1982). The concentrations given in the text are always concentrations of available phosphoryl-group binding sites, corresponding to dimer concentrations (Misset & Robillard, 1982).

Methods. All proton NMR experiments were carried out at 360 MHz on a Bruker HX 360 spectrometer, equipped with an Aspect 2000 computer. Concentrations of pyruvate and of 2-oxobutyrate were determined enzymatically with lactate dehydrogenase. Specific experimental conditions employed are given in the figure captions.

Results

Enzymatic Preparation of Pure Z-PEB. Saier et al. (1980) have shown that 2-oxobutyrate can act as a phosphoryl-group acceptor for phosphoenzyme I. When PEP is used as a phosphoryl-group donor for enzyme I, the overall reaction is the transphosphorylation from PEP to 2-oxobutyrate as represented in Scheme I. In principle, both the *E* and *Z* isomers of PEB could be formed in such a reaction. The two isomers can easily be distinguished on the basis of their proton NMR spectra (Duffy et al., 1982). The C-3 proton NMR signal of PEB as a product from reaction Ic is shown in Figure 1, together with the corresponding signals from a mixture of authentic *E*- and *Z*-PEB. It is clear that only one isomer of PEB is formed in the reaction of phosphoenzyme I with 2-oxobutyrate, and comparison of the proton NMR parameters with those of the synthetically prepared pure *E* and *Z* isomers (Duffy et al., 1982) revealed that it is the *Z* isomer. When the *E*/*Z* mixture reacted with enzyme I, using pyruvate as a phosphoryl-group acceptor for phosphoenzyme I (reaction Ic from right to left), the *Z* isomer disappeared and, as expected, the *E* isomer remained. The equilibrium constant of reaction Ic was estimated to be approximately 20, or $\Delta G^\circ = -1.8 \pm 0.3$ kcal/mol, both from the integrated intensities of the NMR signals at chemical equilibrium and from experiments starting with ^{14}C-labeled PEP in which PEP was separated from pyruvate after chemical equilibrium was established [see Hoving et al. (1981)]. Thus, with $\Delta G^\circ = -14.8$ kcal/mol for the hydrolysis of PEP, a value of -13.0 ± 0.3 kcal/mol is obtained for the hydrolysis of *Z*-PEB.

Pyruvate Kinase Catalyzed Enolization of 2-Oxobutyrate. In the preceding section it was shown that *Z*-PEB can act as a substrate for enzyme I. When D or T is used in the enzymatic protonation of the C-3 atom during this reaction, the stereochemical course of the proton-transfer step can be es-

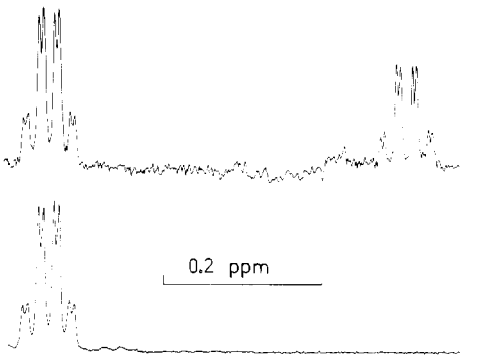
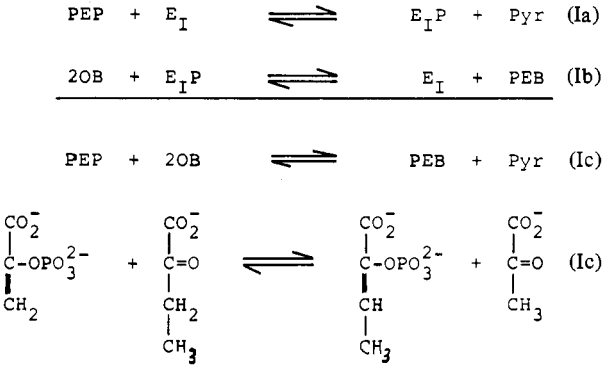


FIGURE 1: C-3 proton NMR signals from PEB at approximately −6.6 ppm: (upper spectrum) mixture of *Z*-PEB (downfield signal, J_{H-H} = 7.1 Hz, J_{H-P} = 2.5 Hz) and *E*-PEB (upfield signal, J_{H-H} = 7.4 Hz, J_{H-P} = 1.9 Hz); (lower spectrum) product of reaction Ic (J_{H-P} = 2.5 Hz, J_{H-H} = 7.1 Hz). Reaction conditions were 100 mM PEP + 500 mM 2-oxobutyrate + 5 μM enzyme I, in 100 mM potassium phosphate buffer in H_2O, pH 7.5, containing 15 mM $MgCl_2$ and 0.5 mM DTT, incubated for 4 h at 37 °C.

Scheme I: Transphosphorylation from PEP to 2-Oxobutyrate



[2] The stereochemical nomenclature used is according to Hanson (1966) (*R*, *S*, *pro-R*, *pro-S*, *re*, *si*) and to Blackwood et al. (1968) (*Z*, *E*), and is illustrated in Scheme III.

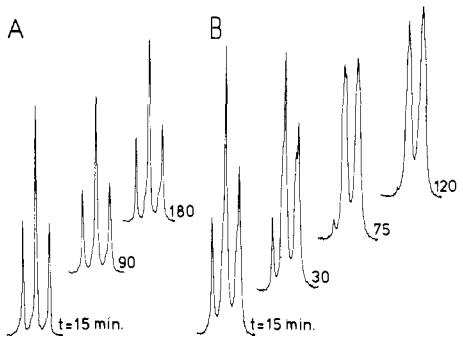
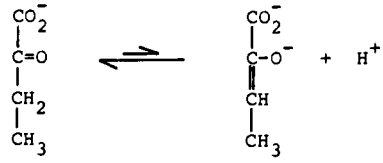


FIGURE 2: Methyl proton NMR signal from 2-oxobutyrate (1.7 ppm, J_{H-H} = 7.0 Hz) as a function of time, in the absence (A) and presence (B) of 1 mg/mL pyruvate kinase. Experimental conditions were 16 mM 2-oxobutyrate in 100 mM potassium phosphate buffer in D_2O, pH 7.5, containing 100 mM KCl and 20 mM $MgCl_2$, incubated at room temperature.

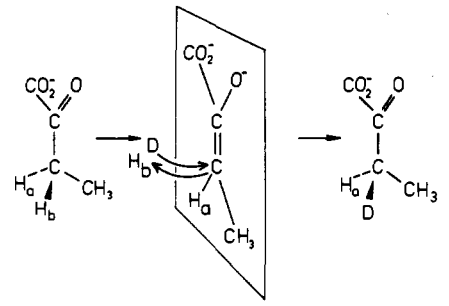
Scheme II: Enolization of 2-Oxobutyrate



tablished by determining the chirality of the resulting 2-oxobutyrate. If 100% of the hydrogen isotope (e.g., D) is used instead of only a trace amount, the chirality of 2-oxobutyrate can be determined by decarboxylation and by measuring the optical rotation of the resulting propionic acid (Adlersberg et al., 1977).

In this section, a simpler and more sensitive method to determine the chirality of C-3 isotopically substituted 2-oxobutyrate will be presented. The method is based on the pyruvate kinase catalyzed enolization of 2-oxobutyrate. Rose (1960) has shown that, in the presence of ATP or a substitute such as phosphate, pyruvate kinase catalyzes the enolization not only of pyruvate but also of a number of analogues including 2-oxobutyrate (see Scheme II). When this reaction was carried out in a phosphate buffer in D_2O, we found that only one of the two C-3 protons of 2-oxobutyrate was exchanged with solvent. This effect can be seen most clearly from the methyl proton NMR signal. In Figure 2, this NMR signal of 2-oxobutyrate is shown as a function of time in the absence (A) and presence (B) of pyruvate kinase in a phosphate buffer in D_2O. Clearly, incubation with pyruvate kinase causes the triplet structure to collapse into a doublet, demonstrating that one of the two C-3 protons is replaced by a deuteron. This doublet does not change into a singlet over the time course of the experiment, showing that the second C-3 proton is not exchanged.[3] This result demonstrates that two processes must take place stereospecifically. The enolization must proceed via a single isomer of the enolate, and the deprotonation must take place specifically from one face of the molecule. The pyruvate kinase catalyzed deprotonation of 2-oxobutyrate proceeds solely via the *Z* isomer of the enolate.[4]

Scheme III: Pyruvate Kinase Catalyzed Enolization of 2-Oxobutyrate[a]



[a] H_a and H_b are the *pro-S* and *pro-R* protons, respectively. The proton exchange occurs at the *2si,3re* face of the *Z* isomer of the enolate. The product of the exchange reaction is 2-oxo-[3(*R*)-H,D]butyrate.

Rose (1970) has shown that, in the reaction with PEP and ADP, pyruvate kinase protonates the intermediate enolate of pyruvate specifically from the *2si* face. Here we have obtained evidence that the deprotonation of 2-oxobutyrate also takes place specifically from one face of the enolate, and on the basis of Rose's results with pyruvate, the *2si,3re* face of the *Z* enolate is the most likely face of protonation. Thus, the stereochemical course of the pyruvate kinase catalyzed enolization of 2-oxobutyrate can be represented as in Scheme III. According to this scheme, the H_a proton is not accessible, and only H_b, the *pro-R* proton, is exchanged. The stereospecificity of this exchange reaction allows a discrimination between the two enantiomeric C-3 protons of 2-oxobutyrate. In the next section this reaction will be used to establish the chirality of 2-oxo-[3-H,D]butyrate produced in the reaction of *Z*-PEB with enzyme I.

The stereospecific exchange reaction described above can also be used to generate the pure *R* and *S* enantiomers of 2-oxo[3-H,D]butyrate. From Scheme III it can be seen that the exchange of 2-oxo[3-H_2]butyrate in phosphate/D_2O yields pure 2-oxo[3(*R*)-H,D]butyrate. The opposite enantiomer is obtained from the exchange of 2-oxo[3-D_2]butyrate in phosphate/H_2O. The deuterated 2-oxobutyrate required for this reaction can easily be prepared by the nonenzymatic enolization of 2-oxobutyrate. As can be seen from Figure 2A, 2-oxobutyrate undergoes slow enolization in the absence of enzyme and in D_2O becomes deuterated at C-3. The deuteration is complete within 24 h at 37 °C in the presence of 10 mM $MgCl_2$.

Stereospecificity of PEB Protonation in the Reaction with Enzyme I. The results presented in the previous two sections enable us to design experiments to establish the stereochemical course of the proton transfer during the reaction of *Z*-PEB with enzyme I. The pure *Z* isomer of PEB was prepared via the enzyme I catalyzed transphosphorylation from PEP to 2-oxobutyrate (Scheme I). PEB was separated from pyruvate, 2-oxobutyrate, and enzyme as previously described for the separation of PEP from pyruvate and enzyme (Hoving et al., 1981), except that the Dowex columns were eluted with solutions KCl instead of LiCl. The purified *Z*-PEB was subsequently used as a substrate for enzyme I, yielding phosphoenzyme I and 2-oxobutyrate. Pyruvate was added as a phosphoryl-group acceptor for phosphoenzyme I, so that the overall reaction was essentially the reverse of the transphosphorylation reaction represented in Scheme I. By carrying out the reaction in D_2O and using C-3 deuterated pyruvate as a phosphoryl-group acceptor, it was ascertained that enzyme I transferred a deuteron to PEB in the forward reaction, yielding 2-oxo[3-H,D]butyrate (see Scheme IVa). On the

[3] Note that the deuteration at C-3 not only changes the fine structure of the methyl proton resonance but also causes a small upfield shift. This shift is approximately 0.01 ppm for one deuteron at C-3 and 0.02 ppm for two deuterons at C-3. At 360 MHz, these shifts are approximately $^1/_2J$ and *J*, respectively, causing the doublet from 2-oxo[3-H,D]butyrate to overlap with the two upfield peaks of the triplet from 2-oxo[3-H_2]-butyrate and the singlet from 2-oxo[3-D_2]butyrate with the most upfield peak of the triplet and doublet.

[4] The phosphorylation of 2-oxobutyrate from ATP by pyruvate kinase yields only (*Z*)-PEB (unpublished results).

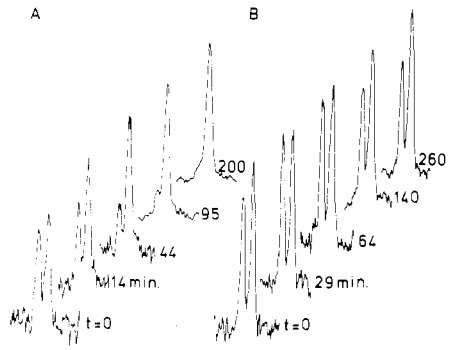
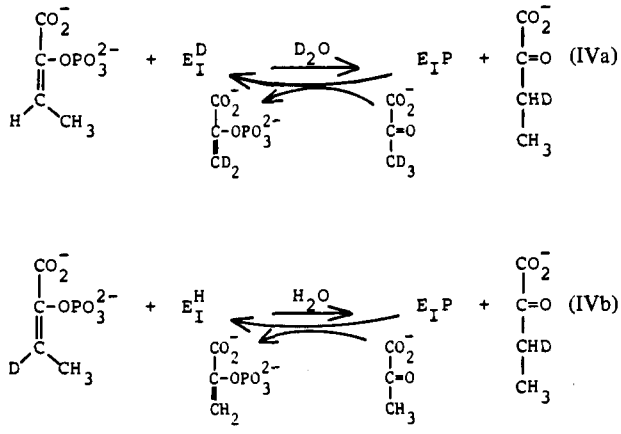


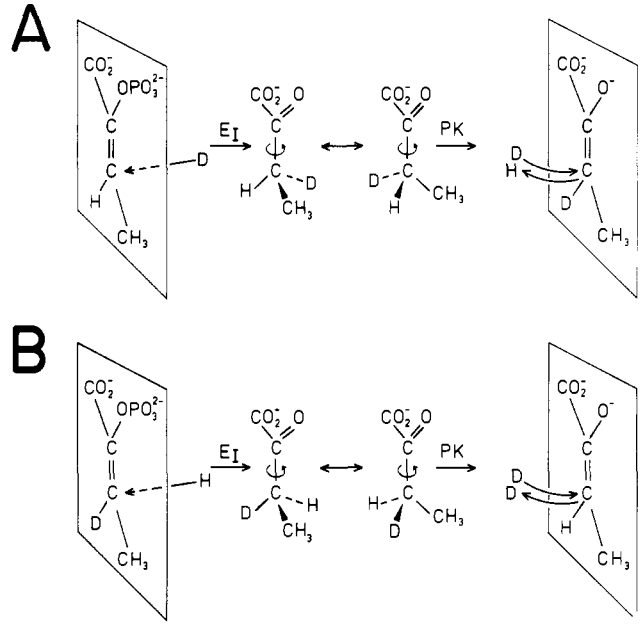
FIGURE 3: Methyl proton NMR signals from 2-oxobutyrate (1.7 ppm, J_{H-H} = 7.0 Hz), as a function of time, in 100 mM potassium phosphate buffer in D_2O, pH 7.5, containing 100 mM KCl, 25 mM $MgCl_2$, and 1 mg/mL pyruvate kinase, at room temperature. (A) 2-Oxobutyrate from reaction IVa. 10 mM [3(*Z*)-H]PEB + 20 mM [3-D_3]pyruvate in 75 mM potassium phosphate buffer in D_2O, pH 7.5, containing 500 mM KCl, 20 mM $MgCl_2$, 1 mM DTT, and 15 μM enzyme I, were incubated at 37 °C for 75 min. The sample (0.5 mL) was then diluted 5-fold in D_2O, put on a 0.5-mL Dowex AG1X2 50–100 mesh column, washed with 1 mL D_2O, and eluted with 100 mM potassium phosphate buffer in D_2O, pH 7.5, containing 100 mM KCl. The third 0.4-mL fraction was taken, $MgCl_2$ and pyruvate kinase were added, and the NMR spectra were recorded as a function of time at room temperature. (B) 2-Oxobutyrate from reaction IVb. 10 mM [3-(*Z*)-D]PEB + 20 mM [3-H_3]pyruvate in 75 mM potassium phosphate buffer in H_2O, pH 7.5, containing 500 mM KCl, 20 mM $MgCl_2$, 1 mM DTT, and 15 μM enzyme I, were incubated at 37 °C for 75 min. The product was then treated as described for (A).

Scheme IV: Reactions of Protonated and Deuterated *Z*-PEB with Enzyme I



basis of previous results (Hoving et al., 1981), it is to be expected that intermolecular transfer of a deuteron from pyruvate to PEB takes place. However, a slow exchange of deuterons on enzyme I with solvent cannot be excluded, and in order to prevent the uptake of protons via such a process, the reaction was carried out in D_2O. The same reaction was also carried out with C-3 deuterated *Z*-PEB in H_2O, using C-3 protonated pyruvate as a phosphoryl-group acceptor (Scheme IVb). Stereospecificity in the proton (deuteron) transfer, if any, would result in the two opposite enantiomers of 2-oxo-[3-H,D]butyrate in these two reactions. The enantiomeric composition of the 2-oxo[3-H,D]butyrate was then determined by subjecting it to pyruvate kinase catalyzed enolization in phosphate/D_2O (see Scheme III). The methyl proton NMR signals of 2-oxobutyrate from reactions IVa and IVb are shown as a function of time upon incubation with pyruvate kinase in phosphate/D_2O in parts A and B of Figure 3, respectively. In both cases, a doublet is observed at $t = 0$, because in both cases there is only a single C-3 proton coupled to the methyl protons. In Figure 3A this C-3 proton is rapidly exchanged

Scheme V: Stereochemical Representation of Results in Figure 3



for a solvent deuteron,[3] and comparison of this result with Scheme III reveals that in the 2-oxo[3-H,D]butyrate from reaction IVa the proton is at the position of H_b. This identifies the 2-oxo[3-H,D]butyrate from reaction IVa as the *S* enantiomer. In Figure 3B, the proton is at the position of H_a, which is not accessible to the pyruvate kinase catalyzed exchange, and thus, the 2-oxo[3-H,D]butyrate from reaction IVb is identified as the *R* enantiomer. The stereochemical interpretation of these results is represented in Scheme V, and the conclusion is that enzyme I protonates *Z*-PEB stereospecificity at the *2re,3si* face.

The extent to which this proton transfer is stereospecific can be estimated after careful inspection of the data in Figure 3. Figure 3B shows a slow change of doublet structure into singlet[3] (note that for the determination of the percentage of singlet, the singlet part of the signal has to be compared with the total intensity of the remaining doublet). This slow change must be due to the nonenzymatic enolization of 2-oxobutyrate as shown in Figure 2A. It cannot be caused by a partial nonstereospecificity of the enzyme I reaction, because in that case a rapid change of the corresponding part of the doublet into singlet would have been observed. Furthermore, if the enzyme I reaction were only partially stereospecific, part of the doublet structure in Figure 3A would have remained after 200 min. The data in Figure 3 therefore allow the conclusion that the stereospecificity of the proton transfer by enzyme I must be nearly 100%.

The whole procedure described above was inverted in order to establish the stereochemical course of the proton transfer in the reverse direction, the reaction of phosphoenzyme I with 2-oxobutyrate. Again, two complementary reactions were carried out. In the first one, 2-oxo[3-H_2]butyrate was treated with pyruvate kinase in phosphate/D_2O, yielding 2-oxo[3-(*R*)-H,D]butyrate. This was subsequently used as a substrate for phosphoenzyme I with PEP as the phosphoryl-group donor for enzyme I (see Scheme I). Since only the *Z* isomer of PEB is formed in this transphosphorylation reaction, it is precisely the reverse of the first step in reaction Vb. The complementary experiment was carried out with 2-oxo[3(*S*)-H,D]butyrate, obtained from the pyruvate kinase catalyzed exchange of 2-oxo[3-D_2]butyrate in phosphate/H_2O. On the basis of the preceding results, it should be expected that, where pyruvate

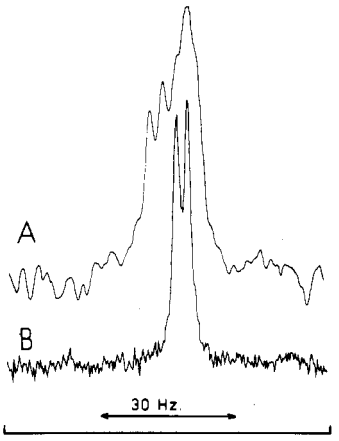


FIGURE 4: Methyl proton NMR signals from *Z*-PEB (2.4 ppm). (A) *Z*-PEB from the reverse of reaction Va. 4.6 mM 2-oxo[3-D_2]butyrate in 130 mM potassium phosphate buffer in H_2O, pH 7.5, containing 100 mM KCl, 16 mM $MgCl_2$, and 1 mg/mL pyruvate kinase, was incubated at room temperature for 2 h. The sample (1.5 mL) was then diluted 2-fold in D_2O, put on a 0.75-mL Dowex AG1X2 50–100 mesh column, washed with 2 mL of D_2O, and eluted with 100 mM potassium phosphate buffer in D_2O, pH 7.5, containing 100 mM KCl. The third 0.5-mL fraction, containing 1.5 mM 2-oxobutyrate, was taken, 10 mM PEP, 25 mM $MgCl_2$, 1 mM DTT, and 4 μM enzyme I were added, the sample was then incubated at 37 °C for 75 min, and the NMR spectrum was run. (B) *Z*-PEB from the reverse of reaction Vb. 4.6 mM 2-oxo[3-H_2]butyrate in 130 mM potassium phosphate buffer in D_2O, pH 7.5, containing 100 mM KCl, 16 mM $MgCl_2$, and 1 mg/mL pyruvate kinase, was incubated at room temperature for 2 h. The sample was further treated as described for (A). A 1-Hz line broadening in the NMR spectrum was applied in (A) to improve signal to noise, because much less product was formed than in (B), due to a kinetic H/D isotope effect.

kinase has replaced one C-3 proton in 2-oxo[3-H_2]butyrate by a deuteron, phosphoenzyme I will abstract the remaining proton to yield [3(*Z*)-D]PEB. In the case where pyruvate kinase has replaced one deuteron on 2-oxo[3-D_2]butyrate by a proton, it is to be expected that phosphoenzyme I will abstract the remaining deuteron, yielding [3(*Z*)-H]PEB. The methyl proton NMR signals of the *Z*-PEB formed in these two reactions are shown in Figure 4. A doublet of doublets is expected for [3(*Z*)-H]PEB (J_{H_3-H} = 7.0 Hz; J_{H_3-P} = 2.5 Hz) and only one doublet for [3(*Z*)-D]PEB (J_{H_3-P} = 2.5 Hz). Figure 4B shows the methyl proton resonance of *Z*-PEB obtained from the reverse of reaction Vb (starting with 2-oxo-[3-H_2]butyrate instead of 2-oxo[3-H,D]butyrate). [3(*Z*)-D]PEB is the expected product, and indeed, the methyl proton resonance does not exhibit the 7.0-Hz coupling with a C-3 proton. In Figure 4A, the methyl proton resonance of *Z*-PEB obtained from the reverse of reaction Va is shown. In this case, abstraction of the C-3 deuteron by phosphoenzyme I is expected, yielding [3(*Z*)-H]PEB and resulting in a doublet of doublets in the NMR spectrum. The signal in Figure 4A shows that, although most of the product is indeed [3(*Z*)-H]PEB, some [3(*Z*)-D]PEB is formed as well. The same line shape was obtained from an 80%/20% mixture of [3(*Z*)-H]- and [3(*Z*)-D]PEB. Since both reactions with phosphoenzyme I were carried out in phosphate/D_2O in the presence of 20 mM $MgCl_2$ at 37 °C for 75 min, some nonenzymatic and nonstereospecific deuteration at C-3 of 2-oxobutyrate occurs (see above). In Figure 4B, phosphoenzyme I leaves the deuteron in 2-oxo[3-H,D]butyrate on PEB, and therefore, the nonenzymatic deuteration at C-3 of 2-oxobutyrate during the reaction is not observed in the methyl proton spectrum of PEB. In Figure 4A, however, the C-3 proton in 2-oxo[3-H,D]-butyrate is expected to remain in PEB, and in this case, the nonenzymatic enolization of 2-oxobutyrate will partly replace

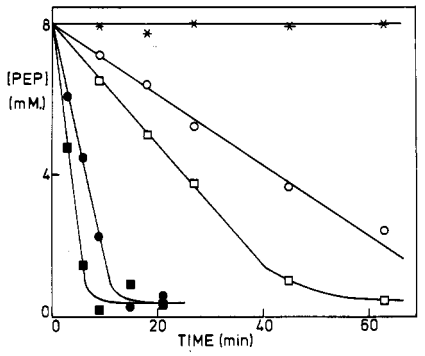


FIGURE 5: Conversion of [^{14}C]PEP into pyruvate in the reaction with enzyme I and excess 2-oxo[3-H_2]butyrate (filled symbols) or 2-oxo[3-D_2]butyrate (open symbols) as a phosphoryl-group acceptor for phosphoenzyme I. Experimental conditions were 8 mM PEP + 80 mM (○, ●) or 160 mM (□, ■) 2-oxobutyrate, in 100 mM potassium phosphate buffer in H_2O, pH 7.5, containing 20 mM $MgCl_2$ and 4 μM enzyme I, incubated at 37 °C. (*) Control without enzyme.

this proton by a solvent deuteron prior to the reaction with phosphoenzyme I, giving rise to the 20% [3(*Z*)-D]PEB. Therefore, both experiments lead to the conclusion that phosphoenzyme I only abstracts the C-3 proton from 2-oxobutyrate that is not accessible to the pyruvate kinase catalyzed exchange. This result is in agreement with the stereochemistry as determined in the forward reaction (see Scheme V).

These results show that enzyme I protonates *Z*-PEB stereospecificially at the 2*re*,3*si* face, yielding 2-oxobutyrate with the enzyme I derived proton at the *pro-S* position. In the reverse reaction, phosphoenzyme I abstracts only the *pro-S* proton from C-3 of 2-oxobutyrate, and pure *Z*-PEB is the product.

Kinetic H/D Isotope Effect. A substantial kinetic H/D isotope effect was observed during the stereochemical experiments in which the *R* and *S* enantiomers of 2-oxo[3-H,D]-butyrate were used as substrates for phosphoenzyme I (see previous section). A series of experiments was performed to quantitate this effect. 2-Oxo[3-H_2]butyrate and 2-oxo[3-D_2]butyrate were used as substrates for phosphoenzyme I, and both kinetic experiments were carried out in H_2O. ^{14}C-Labeled PEP was used to rephosphorylate enzyme I in situ, and the rate of conversion of [^{14}C]PEP to [^{14}C]pyruvate was measured as described previously (Hoving et al., 1981). The results from two sets of two experiments are shown in Figure 5. In all four experiments, a 10-fold or 20-fold excess of 2-oxobutyrate over PEP was used, and as can be seen, the disappearance of PEP was linear as a function of time until at least 90% conversion. This indicates that the phosphorylation of enzyme I from PEP was rapid and, consequently, that the reaction of phosphoenzyme I with 2-oxobutyrate was the rate-determining reaction until at least 90% of the PEP was converted. Using steady-state approximation, the reaction rate in units of millimolar per minute can be expressed as an overall second-order rate constant multiplied by the product of 2-oxobutyrate and phosphoenzyme I concentrations. If the steady-state concentration of phosphoenzyme I remains constant in going from 2-oxo[3-H_2]butyrate to 2-oxo[3-D_2]butyrate, then the data in Figure 5 directly give the H/D isotope effect in the second-order rate constant. In order to check this, two concentrations of both 2-oxo[3-H_2]butyrate and of 2-oxo[3-D_2]-butyrate were used, and in both cases, the reaction was almost proportional to the concentration of 2-oxobutyrate. This shows that phosphoenzyme I was not saturated with 2-oxobutyrate in either case and, hence, that the concentrations of phosphoenzyme I were equal in the reactions with protonated and deuterated 2-oxobutyrate. Therefore, the isotope effect of 6.8

in Figure 5 is the H/D isotope effect in the second-order rate constant for the reaction of phosphoenzyme I with 2-oxobutyrate.

Discussion

Stereospecificity of Enzyme I. Since the protonation of the free enolate of 2-oxobutyrate in solution is not a stereospecific process, its stereospecificity during the reaction of PEB with enzyme I as demonstrated in this study shows that this step takes place on the enzyme. This confirms our earlier conclusions from kinetic experiments with PEP/pyruvate (Hoving et al., 1981). The data presented in this paper show a high degree of stereospecificity not only for the transfer of a proton from enzyme I to PEB but also for the transfer of a deuteron, which, from the isotope-effect studies, must be at least a factor of 6.8 slower than proton transfer. Although these data do not necessarily imply that phosphoryl-group and proton transfers take place in a concerted manner, the fact that enzyme I cannot release the enolate form of the product, at any significant rate, demonstrates the essential role that this proton-transfer step plays in the mechanism of this enzyme. By contrast, results from similar studies with pyruvate kinase showed that proton transfer to PEB by this enzyme is less than 100% stereospecific (Aldersberg et al., 1977) and that deuteron transfer is substantially less stereospecific than proton transfer (T. Nowak et al., unpublished results).

On the basis of the observation that, in the presence of phosphate and Mg^{2+}, pyruvate kinase catalyzes the exchange of the *pro-R* proton at C-3 of 2-oxobutyrate with solvent (see also below), it can be concluded from Figure 3 and Scheme V that enzyme I protonates *Z*-PEB at the *2re,3si* face. It seems justified to assume that the extra methyl group in PEB as compared with PEP will not affect the orientation of the catalytic base on the enzyme relative to the face of the substrate and that in the case of PEP the protonation will also take place at the *2re* face. It should be noted, however, that the extent of stereospecificity, unlike the sign of it, may in some cases vary with the substrate analogue used (Adlersberg et al., 1977; T. Nowak et al., unpublished results).

During the reaction of phosphoenzyme I with 2-oxobutyrate, two processes take place stereospecifically. First, only the *Z* isomer of PEB is formed, as is demonstrated in Figure 1. This result is in agreement with the observation that the *E* isomer of PEB is not a substrate for enzyme I. Second, the deprotonation of 2-oxobutyrate is stereospecific: the *pro-S* proton, the one that is not exchanged with solvent by pyruvate kinase, is abstracted by phosphoenzyme I, as demonstrated by the data in Figure 4. This result is consistent with the stereochemistry of the proton transfer as detected during the reaction of *Z*-PEB with enzyme I (compare Scheme V). In the reaction of 2-oxo[3(*S*)-H,D]butyrate with phosphoenzyme I, the C-3 deuteron is abstracted by the enzyme, even though the H/D isotope effect is at least a factor of 6.8. This indicates that the stereochemical preference on the enzyme, both for exclusively the *Z* isomer of the enolate and for proton transfer at the *2re,3si* face of this enolate, must be very strong.

The stereospecificity of enzyme I enables us to generate the pure *Z* isomer of PEB in the transphosphorylation from PEP to 2-oxobutyrate. Since only the *pro-R* proton at C-3 of 2-oxobutyrate is retained in *Z*-PEB, the reaction also provides a means to discriminate between the two enantiomeric C-3 protons of 2-oxobutyrate.

Kinetic Isotope Effects. A H/D isotope effect of 6.8 was found in the second-order rate constant for the reaction of phosphoenzyme I with protonated and deuterated 2-oxobutyrate. This result demonstrates that the step in which proton transfer occurs is strongly, perhaps fully, rate determining in the overall reaction. Proton transfer may or may not be concerted with phosphoryl-group transfer. In case of a nonconcerted mechanism, it appears that proton transfer rather than phosphoryl-group transfer is the rate-limiting step. This, together with the high degree of stereospecificity, then demonstrates that the proton-transfer step is an essential part of the reaction mechanism. The catalytic significance of the proton transfer as demonstrated here would also be consistent with a concerted mechanism.

Stereospecificity of Pyruvate Kinase Catalyzed Enolization of 2-Oxobutyrate. The sign of the stereospecificity of the enzyme I reaction (proton transfer to the *2re,3si* face of *Z*-PEB) as determined in this study fully depends on the "known" stereospecificity of the pyruvate kinase catalyzed enolization of 2-oxobutyrate. In the experiments shown in Figure 3, the chirality of 2-oxo[3-H,D]butyrate formed in the reaction of *Z*-PEB with enzyme I was analyzed by this pyruvate kinase catalyzed enolization process. In the experiments in Figure 4, the substrates for phosphoenzyme I were the *R* and *S* enantiomers of 2-oxo[3-H,D]butyrate formed via the same pyruvate kinase catalyzed enolization reaction. Knowledge of the stereochemistry of this enolization reaction is therefore requisite to a correct interpretation of the results with enzyme I.

The stereochemistry as represented in Scheme III is based upon two processes that must take place in a stereospecific fashion: (i) enolization via the *Z* isomer of the enolate, which is consistent with the observation that only the *Z* isomer of PEB is formed when the deprotonation of 2-oxobutyrate is followed by phosphorylation from ATP, and (ii) protonation/deprotonation at the *2si,3re* face of the enolate, which is in agreement with the stereochemistry of the proton transfer to the enolate of pyruvate in the overall reaction with PEP and ADP (Rose, 1970). However, some doubt about the correctness of Scheme III may be evoked by the observation that, during the pyruvate kinase catalyzed reaction between *Z*-PEB and ADP, the intermediate enolate of 2-oxobutyrate is protonated at the *2si,3re* face with only 70% stereospecificity (Adlersberg et al., 1977). This result immediately raises the question whether the totally stereospecific proton-exchange reaction with phosphate as a cofactor has anything to do with the overall reaction and, therefore, whether the sign of the stereospecificity is the same. As a test of the correctness of Scheme III, the experiment as presented by Adlersberg et al. (1977) was repeated, and the chirality of the product, 2-oxo[3-H,D]butyrate, was analyzed by using the pyruvate kinase catalyzed exchange in phosphate/D_2O, instead of the optical analysis used by those authors. We observed, as did Adlersberg et al. (1977), that the pyruvate kinase catalyzed conversion of [3(*Z*)-H]PEB to 2-oxo[3-H,D]butyrate in the presence of ADP in D_2O was only partially stereospecific. When the corresponding reaction with [3(*Z*)-D]PEB in H_2O was examined, the stereospecificity was higher. This suggests that there are two competing processes, the stereospecific transfer of a proton (or deuteron) from the enzyme to the bound enolate and a nonstereospecific protonation (deuteration) from solution if the enolate intermediate leaves the enzyme surface before being protonated. Since the chirality of the product formed from *Z*-PEB and ADP was the same as that obtained by pyruvate kinase catalyzed exchange of 2-oxobutyrate with phosphate as a cofactor, the correctness of Scheme III is assured. The stereochemistry as represented in Scheme III was further confirmed by proton NMR studies of the resulting *R* and *S* enantiomers of 2-oxo[3-H,D]butyrate

after reduction to 2-hydroxybutyrate with lactate dehydrogenase. These experiments and additional experiments on the stereospecificity of pyruvate kinase with different PEP analogues will be published separately.

Acknowledgments

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Registry No. *Z*-PEB, 31302-64-4; *E*-PEB, 31302-89-3; PEP, 138-08-9; PEP–sugar phosphotransferase, 56941-29-8; PEP–protein phosphotransferase, 37278-17-4.

References

Adlersberg, M., Dayan, J., Bondinell, W. E., & Sprinson, D. B. (1977) *Biochemistry 16*, 4382–4387.
Blackwood, J. E., Gladys, C. L., Loening, K. L., Petrarca, A. E., & Rush, J. E. (1968) *J. Am. Chem. Soc. 90*, 509–510.
Blumberg, K., & Stubbe, J. A. (1975) *Biochim. Biophys. Acta 384*, 120–126.
Bondinell, W. E., & Sprinson, D. B. (1970) *Biochem. Biophys. Res. Commun. 40*, 1464–1467.
Brouwer, M., Elferink, M., & Robillard, G. T. (1982) *Biochemistry 21*, 82–88.
Cornforth, J. W., Redmond, J. W., Eggerer, H., Buckel, W., & Gutschow, Ch. (1969) *Nature (London) 221*, 1212–1213.
Duffy, T. H., Saz, H. J., & Nowak, T. (1982) *Biochemistry 21*, 132–139.
Hanson, K. R. (1966) *J. Am. Chem. Soc. 88*, 2731–2742.
Hays, J. B. (1978) *Bacterial Transport* (Rosen, B. P., Ed.) pp 43–102, Marcel Dekker, New York.
Hoving, H., Lolkema, J. S., & Robillard, G. T. (1981) *Biochemistry 20*, 87–93.
Lüthy, J., Rêtey, J., & Arigoni, D. (1969) *Nature (London) 221*, 1213–1215.
Misset, O., & Robillard, G. T. (1982) *Biochemistry 21*, 3136–3142.
Robillard, G. T. (1982) *Mol. Cell. Biochem. 46*, 3–24.
Robillard, G. T., Dooyewaard, G., & Lolkema, J. S. (1979) *Biochemistry 18*, 2984–2989.
Rose, I. A. (1960) *J. Biol. Chem. 235*, 1170–1177.
Rose, I. A. (1970) *J. Biol. Chem. 245*, 6052–6056.
Saier, M. H., Schmidt, M. R., & Lin, Ph. (1980) *J. Biol. Chem. 255*, 8579–8584.
Stein, R., Schrecker, O., Lauppe, H. F., & Hengstenberg, W. (1974) *FEBS Lett. 42*, 98–100.
Stubbe, J. A., & Kenyon, G. L. (1971) *Biochemistry 10*, 2669–2677.
Stubbe, J. A., & Kenyon, G. L. (1972) *Biochemistry 11*, 338–345.
Waygood, E. B., & Steeves, T. (1980) *Can. J. Biochem. 58*, 40–48.

Reversible Inhibition of the Bacterial Luciferase Catalyzed Bioluminescence Reaction by Aldehyde Substrate: Kinetic Mechanism and Ligand Effects†

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ABSTRACT: The bioluminescence reaction catalyzed by bacterial luciferase from the luminous marine bacterium *Vibrio harveyi* was found to be subject to reversible, chain length dependent inhibition by aldehyde substrate. The stoichiometry of aldehyde to luciferase in the bioluminescence reaction was 1:1; the kinetics of substrate inhibition were consistent with the binding of a second molecule of aldehyde to luciferase to form an enzymatically inactive complex. These findings indicated that aldehyde interactions with bacterial luciferase from *V. harveyi* could not be adequately described by simple Michaelis–Menten kinetics. The binding of *n*-decanal to luciferase and the bioluminescence reaction velocity were dependent on buffer composition and concentration. Phosphate binding to luciferase reduced enzyme affinity for binding a second molecule of aldehyde; the reciprocal effect was also observed. The existence of a reversible complex between the aldehyde substrate and luciferase suggested that the reaction in vitro did not require ordered binding of $FMNH_2$ and aldehyde, in contrast to the commonly depicted kinetic model implying ordered substrate binding. The data presented here, along with recent observations [Holzman, T. F., & Baldwin, T. O. (1981) *Biochemistry 20*, 5524–5528; Holzman, T. F., & Baldwin, T. O. (1982) *Biochemistry 21*, 6194–6201], suggest a kinetic model for the luciferase-catalyzed reaction in which the order of substrate binding is random.

Bacterial luciferase is a heterodimeric flavin monooxygenase which catalyzes the oxidation of reduced riboflavin 5′-phosphate ($FMNH_2$)[1] and long-chain aldehydes to yield blue-green light [see Ziegler & Baldwin (1981) for a recent review]. The conversion of the aldehyde to acid in the reaction in vitro has been demonstrated (Shimomura et al., 1972; Dunn et al., 1973; McCapra & Hysert, 1973; Vigny & Michelson, 1974). There is evidence for the participation of tetradecanal in the reaction in vivo (Ulitzur & Hastings, 1978, 1979). The aldehyde alkyl chain lengths for maximal activity in vitro have been determined for luciferases from a variety of bacterial species [see

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; BSA, bovine serum albumin; DPDA, 2-(2,4-dichloro-6-phenylphenoxy)-*N*,*N*-diethylamine; DPEA, 2-(2,4-dichloro-6-phenylphenoxy)ethylamine; DϕPA, 2,2-diphenylpropylamine; DϕPA–Sepharose, 2,2-diphenylpropylamine–bis(oxirane)–Sepharose; DTE, dithioerythritol; FMN, riboflavin 5′-phosphate; $FMNH_2$, reduced FMN; LU, light units; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TLU, total light units; $NaDodSO_4$, sodium dodecyl sulfate.

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