Monatshefte für Chemie 113, 999—1004 (1982)

Monatshefte für Chemie
Chemical Monthly

Reaction of Pyridoxal 5′-Phosphate with TRIS

Michael D. Davis, Dale E. Edmondson, and **Donald B. McCormick***

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

(Received 28 December 1981. Accepted 28 February 1982)

Kinetic and equilibrium studies are presented on the reaction of pyridoxal 5′-phosphate with *Tris*. Upon raising the *pH* from 7.3 to 9.3 at 25 °C, the second-order rate constant increases from 1.05 $M^{-1} s^{-1}$ to 3.79 $M^{-1} s^{-1}$, whereas the apparent dissociation constant varies from 2 to 8 mM. These results demonstrate the significance of this reaction when *Tris* buffer is used in studies of pyridoxal 5′-phosphate-dependent enzymes.

(Keywords: Coenzymes; Pyridoxal 5′-phosphate; Tris)

Reaktion von Pyridoxal-5′-phosphat mit TRIS

Es werden Untersuchungen über die Kinetik und das Gleichgewicht der Reaktion von Pyridoxal-5′-phosphat mit *TRIS* vorgelegt. Wenn der *pH* bei 25 °C von 7,3 auf 9,3 erhöht wird, steigt die Geschwindigkeitskonstante zweiter Ordnung von 1,05 $M^{-1} s^{-1}$ auf 3,79 $M^{-1} s^{-1}$, während die scheinbare Dissoziationskonstante sich im Bereich von 2 bis 8 mM verändert. Die Ergebnisse zeigen, welche Bedeutung diese Reaktion für Untersuchungen von Pyridoxal-5′-phosphat-abhängigen Enzymen bei Verwendung von *TRIS*-Puffer hat.

Introduction

Although *Tris* has been used as a standard buffer in biochemical research for over 20 years[1], its use with systems containing pyridoxal 5′-phosphate can result in complications from the nonenzymatic reaction of the primary amine function with the aldehyde moiety of the coenzyme[2,3]. The *Schiff*'s base formed on reaction of *Tris*-Pyridoxal 5′-phosphate has a dissociation constant in the millimolar range[2], and results in a red-shifted adsorbance spectrum[3] as compared with the spectrum of free coenzyme. This reaction has been utilized to diminish product inhibition that occurs during the reaction catalyzed by

0026-9247/82/0113/0999/$ 01.20

pyridoxamine (pyridoxine) 5′-phosphate oxidase (EC 1.4.3.5). The concomitant color change allows spectrophotometric monitoring of product release (414 nm)[4,5] which, however, overlaps the absorbance of the FMN (448 nm) associated with the enzyme and can result in complications when the flavin is monitored spectrally during catalytic turnover. This communication describes the kinetic and equilibrium parameters of the *Tris*-pyridoxal 5′-phosphate interaction so as to provide a rationale for minimization of any interference of the non-enzymic reaction with the enzyme-catalyzed reaction.

Experimental

Pyridoxal 5′-phosphate and *Tris* were purchased from Sigma Chemical Co. Optical spectra were recorded on a Varian 219 spectrophotometer. Kinetic experiments were performed on an Update Instrument stopped-flow spectrometer, Model 731, interfaced to a North Star Horizon computer, using the hardware and software provided by On-Line Instrument Systems, Jefferson, GA. Equilibrium studies were performed spectrophotometrically at 278 nm and 25 °C in the presence of 0.1 M NaCl. Titration data were analyzed by both *Benesi-Hildebrand*[6] and *Scatchard* procedures[7]. The apparent rate constants (k_{obs}) were computed from the kinetic traces using a single exponential non-linear regression curve-fitting routine. Least square analyses were used for both the calculation of the equilibrium and the kinetic parameters from all linear plots, yielding correlation coefficient values (r) of 0.994 or better[8]. When the kinetic data were to be compared to the corresponding equilibrium data, a constant ionic strength of 0.1 was maintained by the addition of NaCl, and the concentration of *Tris* was kept to 75 mM or less.

Results and Discussion

Three major spectral effects between 500 and 250 nm were observed on raising the *pH* from 7.3 to 9.3 of a pyridoxal 5′-phosphate solution (Fig. 1); these changes are predominantly due to deprotonation of the ring nitrogen ($pK_a = 8.7$)[9]. First, the peak at 390 nm increases in absorbance, i.e., the molar absorbance coefficient is raised from 4,900 to 5,900 $M^{-1}\,cm^{-1}$. Second, a shoulder at approximately 330 nm disappears. Third, a shoulder at 265 nm is formed. Isobestic points at 351 nm ($\varepsilon = 2{,}700\,M^{-1}\,cm^{-1}$) and 280 nm ($\varepsilon = 900\,M^{-1}\,cm^{-1}$) are observed*.

In agreement with previous observations[2–5], the addition of *Tris* to a solution of pyridoxal 5′-phosphate results in a spectrum displaying two major peaks, viz. at 414 nm ($\varepsilon = 5{,}900\,M^{-1}\,cm^{-1}$) and at 278 nm ($\varepsilon = 5{,}600\,M^{-1}\,cm^{-1}$).

---

* For a comprehensive review on the spectral properties of pyridoxal 5′-phosphate and its corresponding *Schiff*'s bases, see Refs.[9,10].

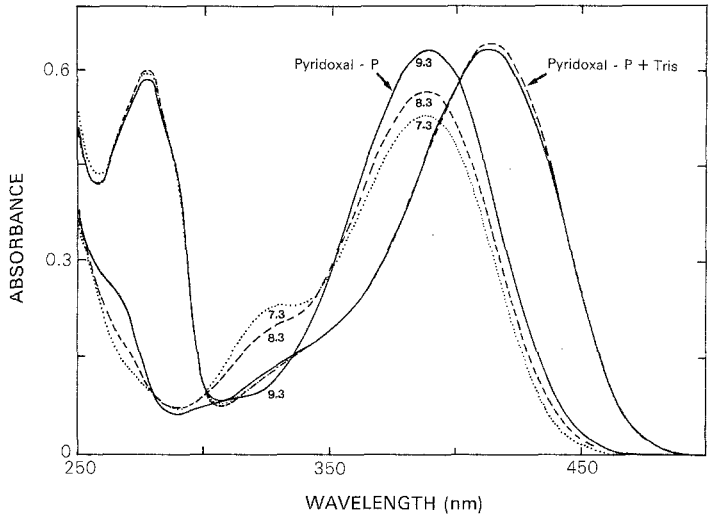

Fig. 1. Spectra of Pyridoxal 5′-phosphate with and without 0.1 M *Tris* at *pH* 7.3 (...), 8.3 (— — —), and 9.3 (———) at 25 °C

In contrast to that observed with pyridoxal 5′-phosphate, the spectrum of the *Tris*-pyridoxal-5′-phosphate *Schiff*'s base is only slightly altered as the *pH* is increased from 7.3 to 9.3. In fact, the spectra at *pH* 7.3 and 9.3 are practically superimposable, whereas that at *pH* 8.3 displays a slightly greater absorbance at 414 nm.

The rate constants for the reaction are defined by the following scheme[11]:

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \underset{k_4}{\overset{k_3}{\rightleftharpoons}} D \tag{1}$$

with A, B, C, and D representing pyridoxal 5′-phosphate, *Tris*, the resulting carbinolamine and the aldimine respectively[12,13]. Using the steady-state assumption[11], the observed rate constant k_{obs} is equal to:

$$\frac{k_1[B](k_3 + k_4) + k_2 k_4}{k_1[B] + k_2 + k_3} \tag{2}$$

However, under the conditions of our experiments, k_{obs} is a linear function of [B], implying $(k_2 + k_3) \gg k_1[B]$. Furthermore, *Jo* et al.[13], studying the reaction of *DL*-alanine and pyridoxal 5′-phosphate by ^{13}C NMR, report that between *pH* 7.1-10.5 the equilibrium between the carbinolamine and the aldimine is shifted far towards the right indicating that $k_3 \gg k_4$ under the conditions of this study. Therefore:

$$k_{obs} = \frac{k_1 k_3}{k_2 + k_3}[B] + \frac{k_2 k_4}{k_2 + k_3} \tag{3}$$

The slope of the plot of k_{obs} versus [*Tris*] is thus $\frac{k_1 k_3}{k_2 + k_3} \equiv k_{forward}$, whereas the y-intercept is $\frac{k_2 k_4}{k_2 + k_3} \equiv k_{reverse}$. The apparent dissociation constant (K_d) is the product of the individual reverse rate constants divided by the product of the individual forward rate constants:

$$\frac{k_2 k_4}{k_1 k_3} = \frac{k_{reverse}}{k_{forward}} \tag{4}$$

The reaction of *Tris* with pyridoxal 5'-phosphate displays a linear correlation between the concentration of *Tris* and the apparent pseudo-first-order rate constant (k_{obs}) at all pH values and temperatures tested (Fig. 2). At pH 8.3 the value of $k_{forward}$ at 13 °C (1.1 $M^{-1} s^{-1}$) is 4.4-fold

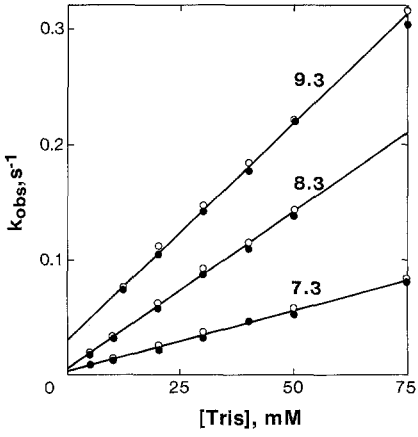

Fig. 2. Effect of *Tris* concentration on the observed rate of aldimine formation. k_{obs} versus [*Tris*] at pH values indicated and 25 °C, ○—○—○ 450 nm, ●—●—● 380 nm

slower than that at 37 °C (5.0 $M^{-1} s^{-1}$), which indicates an approximate 2-fold change per 10 °C increment (not shown). Increasing the pH results in an increase in $k_{forward}$ (Table 1). This is anticipated because it is likely that upon raising the pH, k_1 and k_3 will increase due to the deprotonation of the amino group of *Tris* and the base-catalyzed conversion of the carbinolamine to the aldimine respectively[13]. All plots (Fig. 2A) intercept the ordinate near the origin, which suggests the

Table 1. *Kinetic and equilibrium constants for the reaction of pyridoxal 5′-phosphate with TRIS as a function of pH (the values were obtained as described in the text ± S.D.)*

pH	$k_{forward}$ $M^{-1}s^{-1}$	$10^3 \times k_{reverse}$ s^{-1}	K_d(calc)[a] mM	K_d(obs) mM
7.3	1.05 ± 0.03	3.54 ± 1.02	3.36 ± 0.97	2.96 ± 0.08
8.3	2.73 ± 0.08	5.51 ± 1.85	2.01 ± 0.68	2.18 ± 0.10
9.3	3.79 ± 0.09	30.5 ± 3.00	8.04 ± 0.82	7.74 ± 0.16

[a] Obtained by dividing $k_{reverse}$ by $k_{forward}$.

reverse rate constants are small but have finite values (Table 1). Thus, the formation of the *Tris*-pyridoxal 5′-phosphate aldimine is favored even at relatively low concentrations of *Tris*. Indeed, the apparent dissociation constant (K_d) for the aldimine is on the order of 1 to 10 mM between *pH* 7.3 to 9.3 (Table 1). The dissociation constant decreases by approximately 30% on raising the *pH* from 7.3 to 8.3; however, it increases 4-fold on further raising the *pH* to 9.3. This complex relationship of the dissociation constant with *pH* is not reflective of either $k_{forward}$ or $k_{reverse}$ individually, as both increase with *pH*. There is, however, a reasonable correlation between the equilibrium constants obtained by equilibrium titrations with those calculated using the observed kinetic constants (Table 1).

The results reported here are consistant with both earlier studies of pyridoxal 5′-phosphate with *Tris*[2,14] as well as with similar studies between pyridoxal 5′-phosphate and other amines[10,13,15]. Although *Schiff*'s base formation of *Tris* with pyridoxal has been known for some time[2], the results of this study provide information that will be useful for catalytic studies of pyridoxal 5′-phosphate-dependent enzymes in *Tris* buffer. We find that within one *pH* unit (in either direction) of the pK_a of *Tris*[1], greater than half of the pyridoxal 5′-phosphate "free" in solution will be bound to the buffer at concentrations of *Tris* greater than 10 mM. Furthermore, the *Schiff*'s base formation is relatively slow, i.e., with 50 mM *Tris* at *pH* 8.3, and 25 °C, the forward rate constant is only 8 min^{-1}.

Acknowledgements

We are grateful to *Michael Shapiro* for technical assistance and Dr. *Jung-Do Choi* for helpful discussions. This investigation was supported by Research Grant AM-26746 from the N.I.H. (to *D. B. M.*) and by Research Grant PCM-81-00770 from the N.S.F. (to *D. E. E.*).

References

1 *Good N. E., Izawa S.*, in: Methods in Enzymology (*San Pietro A.*, ed.), Vol. 24 B, pp. 53—63. New York: Academic Press. 1972.
2 *Matsuo Y.*, J. Amer. Chem. Soc. **79**, 2011 (1957).
3 *Pogell B. M.*, J. Biol. Chem. **232**, 761 (1958).
4 *Horiike K., Merrill A. H., jr., McCormick D. B.*, Arch. Biochem. Biophys. **195**, 325 (1979).
5 *Merrill A. H., jr., Kazarinoff M. N., Tsuge H., Horiike K., McCormick D. B.*, in: Methods in Enzymology (*McCormick D. B., Wright L. D.*, eds.), Vol. 62 D, pp. 568—574. New York: Academic Press. 1979.
6 *Benesi H. A., Hildebrand J. H.*, J. Amer. Chem. Soc. **71**, 2703 (1949).
7 *Scatchard G.*, Ann. N. Y. Acad. Sci. **51**, 660 (1949).
8 *Kemeny J. G., Kurtz T. E.*, Basic Programming, 2nd ed., pp. 114—115. New. York: J. Wiley.1971.
9 *Johnson R. J., Metzler D. E.*, in: Methods in Enzymology (*McCormick D. B., Wright L. D.*, eds.), Vol. 18 A, pp. 433—471. New York: Academic Press. 1970.
10 *Metzler C. M., Cahill A., Metzler D. E.*, J. Amer. Chem. Soc. **102**, 6075 (1980).
11 *Strickland S., Palmer G., Massey V.*, J. Biol. Chem. **250**, 4048 (1975).
12 *Kobayashi Y., Makino K.*, Biochim. Biophys. Acta **208**, 137 (1970).
13 *Jo B. H., Nair V., Davis L.*, J. Amer. Chem. Soc. **99**, 4467 (1977).
14 *Federiuk C. S., Shafer J. A.*, J. Biol. Chem. **256**, 7416 (1981).
15 *Schonbeck N. D., Skalski M., Shafer J. A.*, J. Biol. Chem. **250**, 5359 (1975).