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# *N*-Hydroxycarbamate Is the Substrate for the Pyruvate Kinase Catalyzed Phosphorylation of Hydroxylamine†

Paul M. Weiss, Jeffrey D. Hermes, Thomas M. Dougherty, and W. W. Cleland*

ABSTRACT: The true substrate for the pyruvate kinase catalyzed phosphorylation of hydroxylamine at high pH which is activated by bicarbonate is shown to be *N*-hydroxycarbamate, since a lag is seen when the reaction is started by the addition of bicarbonate or hydroxylamine but a burst appears when it is started with a mixture of the two. The lag can be diminished by addition of carbonic anhydrase but not eliminated, showing that $CO_2$ is an intermediate in the formation of the carbamate and that both the formation of $CO_2$ and the subsequent reaction of $CO_2$ with hydroxylamine limit the rate of carbamate formation. The equilibrium constant for the reaction bicarbonate + hydroxylamine ↔ *N*-hydroxycarbamate is 1.33 $M^{-1}$. The product of the phosphorylation decomposes by loss of $CO_2$ to *O*-phosphorylhydroxylamine, which is stable at 25 °C between pH 3 and 11 and has $pK_2 = 5.63$ for the phosphate and $pK_3 = 10.26$ for the amino group.

Kupiecki & Coon (1960) first reported that in the presence of bicarbonate, pyruvate kinase would phosphorylate hydroxylamine. The product of the reaction was identified by Cottam et al. (1968) as *O*-phosphorylhydroxylamine by comparison with the same compound prepared by reaction of phosphoramidate and hydroxylamine (Jencks & Gilchrist, 1965). It was reported by Kupiecki & Coon (1960) that in contrast to the physiological reaction between MgADP and phosphoenolpyruvate, the reaction was activated by $Zn^{2+}$ and not by $Mg^{2+}$. However, Dougherty (1982) has discovered that the reaction is supported by $Mg^{2+}$ at high pH and that $V/K$ decreases a factor of 10 per pH unit below the p$K$ of 9.2 for water in the inner coordination sphere of enzyme-bound $Mg^{2+}$. Since bicarbonate is an obligate activator, and no alternative one has been found despite an extensive search, the question arises whether it activates the reaction by substrate synergism or whether it combines with hydroxylamine to give *N*-hydroxycarbamate according to reaction 1:

$$HCO_3^- \rightleftharpoons CO_2 + OH^- \quad (1a)$$

$$CO_2 + NH_2OH \rightleftharpoons [HO\text{-}\overset{+}{N}H_2\text{-}COO^-] \xrightarrow{OH^-} HO\text{-}NH\text{-}COO^- \quad (1b)$$

*N*-Hydroxycarbamate was suggested to be the substrate by Jencks & Gilchrist (1965), but Cottam et al. (1968), while considering it a possibility, were unable to find any evidence either for or against this hypothesis. The overall equilibrium constant for reaction 1 is independent of pH above pH 7.4,

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received December 13, 1983.* Supported by a grant from the National Institutes of Health (GM 18938).

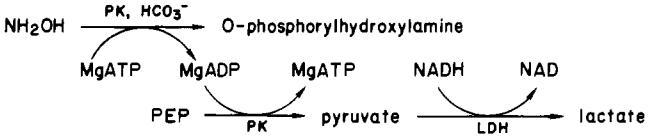
0006-2960/84/0423-4346$01.50/0 

and at the levels of hydroxylamine and bicarbonate we have used, the carbamate level is less than 5% of the bicarbonate and hydroxylamine concentrations. The level of free $CO_2$ varies with pH, however, and is always less than that of the carbamate, so that the rate at which the carbamate forms will decrease as the pH is raised. We have investigated the importance of reaction 1 by beginning the pyruvate kinase catalyzed reaction with either bicarbonate, hydroxylamine, or a mixture of the two in which reaction 1 was at equilibrium.

## Materials and Methods

*Materials.* Pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim as ammonium sulfate suspensions. Phosphoenolpyruvate and NADH were from Sigma, and ATP was from Boehringer Mannheim. $Ba^{13}CO_3$ (97 atom % $^{13}C$) from the Mound Facility of Monsanto (2.5 mL of a 0.60 M solution) was converted to $Na_2CO_3$ by stirring with 1.5 g of Dowex 50-$Na^+$ resin.

*Enzyme Assays.* To determine if a lag is present in the enzymatic reaction, kinetic studies were carried out for the phosphorylation of hydroxylamine by using the self-coupled assay shown below, which is discussed in greater detail by Dougherty (1982):




Each assay was run in 3.0-mL total volume in 1-cm cuvettes by measuring absorbance changes at 340 nm with a Cary 118 spectrophotometer equipped with thermospacers for temperature control. The assay mixtures contained, in order of addition to the cuvette, 100 mM 3-(cyclohexylamino)propanesulfonic acid, pH 10.5, 0.2 mM NADH, 1.0 mM ATP, 0.2 mM phosphoenolpyruvate (PEP), 2.0 mM $MgCl_2$, 100 mM KCl, 0.2 mg of lactate dehydrogenase (LDH), and 0.25 mg of pyruvate kinase (PK). Hydroxylamine and bicarbonate concentrations were varied from 10 to 30 mM. Full-scale sensitivity of 0.2 OD and a chart speed of 0.24 in./min were used. The final pH was determined to be 9.0 ± 0.2 in all cases. The temperature was maintained at 25.0 °C.

*Determination of the pKs of O-Phosphorylhydroxylamine.* A solution containing 100 mM hydroxylamine, 100 mM KCl, 20 mM $MgCl_2$, 10 mM $KHCO_3$, 10 mM ATP, and 250 units/mL pyruvate kinase [free of ammonia in 25 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) buffer, pH 7.0] was incubated at 25 °C pD 9.0, for 24 h. Aliquots were then removed, and the pD was adjusted to the desired value with either NaOD or DCl. The $^{31}P$ NMR spectra of the product mixtures were obtained, and the chemical shift of the phosphorylated product was recorded as a function of pH relative to the chemical shift of 200 mM inorganic phosphate at pH 8.0 in an internal capillary.

*$^{31}P$ and $^{13}C$ NMR.* The $^{31}P$ (81.0 MHz) and $^{13}C$ (50.1 MHz) NMR experiments were performed on a Nicolet NT-200 Fourier transform spectrometer operating in the quadrature detection mode and equipped with a 12-mm broad-band probe for $^{31}P$ and a 5-mm fixed-tune probe for $^{13}C$. For $^{31}P$ spectra, the sample solution (4.0 mL) was contained in a 10-mm NMR tube fitted with a glass capillary inserted through the vortex plug containing 25 μL of 200 mM phosphate at pH 8.0 to serve as an internal reference (0 ppm). $D_2O$ (99.8 atom % D from Bio-Rad) was used for field lock, and all spectra were broad-band proton decoupled in a bilevel mode to prevent sample heating. Acquisition parameters included a 5000-Hz sweep width, 20-μs (75°) pulse width, 1.8-s repetition time, and 8K block size. Five hundred transients were accumulated at each pH before Fourier transformation of the free induction decay.

For $^{13}C$ spectra, the sample solution (0.5 mL) was contained in a 5-mm NMR tube. An external *p*-dioxane standard was used as reference (67.475 ppm). $D_2O$ (80% in the NMR tube) was used for field lock, and bilevel proton decoupling was utilized. Acquisition parameters included a 12000-Hz sweep width, 3–7-μs (36–84°) transmitter pulse width, 3.3–60.3-s repetition time, and 8K block size. Typically 800 transients were accumulated before Fourier transformation of the free induction decay.

*Data Analysis.* Rate constants for lags or bursts in reaction time courses were determined from half-times for achievement of final steady-state velocities. Half-times were determined from recorder tracings of product formation (represented by NADH disappearance in this case) vs. time by (a) drawing a line asymptotic to the final linear steady-state rate, (b) constructing a line parallel to this asymptote passing through the origin at time = 0, and (c) constructing a third parallel line halfway between the first two. The intersection point of this middle line and the recorder tracing defines the half-time for the burst or lag phase of the reaction, and the rate constant is 0.693 divided by the half-time.

The equilibrium constant for *N*-hydroxycarbamate formation was determined from the equation

$$K_{eq} = \frac{1 + R}{R[(1 + R)N - B]} \tag{2}$$

where $R$ is the [bicarbonate]/[*N*-hydroxycarbamate] ratio at equilibrium determined from peak areas in the $^{13}C$ NMR spectrum and $N$ and $B$ are initial concentrations of hydroxylamine and bicarbonate, respectively, added to the reaction.

The $^{31}P$ NMR chemical shifts of *O*-phosphorylhydroxylamine as a function of pH were fitted to eq 3:

$$Y = \frac{YL[H^2/(K_2K_3)] + YM(H/K_3) + YH}{1 + H/K_3 + H^2/(K_2K_3)} \tag{3}$$

where $Y$ is the $^{31}P$ NMR chemical shift at hydrogen ion concentration $H$ and $YL$, $YM$, and $YH$ are the low-, middle-, and high-pH plateau values of the chemical shift, respectively.

## Results and Discussion

*Equilibrium Constant for N-Hydroxycarbamate Formation.* In the presence of hydroxylamine and [$^{13}C$]bicarbonate, a resonance at 167.85 ppm due to *N*-hydroxycarbamate was seen in the $^{13}C$ NMR spectrum in addition to that of bicarbonate at 161.98 ppm. The ratio of the areas of these peaks was determined and used to determine $K_{eq}$ as shown in Table I. The relaxation rates of bicarbonate and the carbamate appear to be similar, since the peak ratio was not a function of pulse angle or delay time up to 60 s. The average $K_{eq}$ value was 1.33 ± 0.19 $M^{-1}$, corresponding to 532 μM carbamate in the presence of 20 mM each of bicarbonate and hydroxylamine.

*Lags and Bursts in Reaction Time Courses.* Proof that *N*-hydroxycarbamate was the actual substrate came from observing lags in the reaction time course when reaction was started with either bicarbonate or hydroxylamine (Figure 1). Carbonic anhydrase eliminated most, but not all, of the lag. When hydroxylamine and bicarbonate were preincubated for 20 min and added to start the reaction, a burst was seen as the level of *N*-hydroxycarbamate decayed to its new equilibrium value after dilution into the reaction mixture. The rate constants for these changes are shown in Table II.

Table I: Equilibrium Constant for Formation of *N*-Hydroxycarbamate

| initial $[HCO_3^-]$ (M) | initial $[NH_2OH]$ (M) | peak ratio[a] | $K_{eq}$ | repetition time (s) | pulse angle (deg) |
|---|---|---|---|---|---|
| 0.24 | 3.0 | 0.26 | 1.38 | 3.3 | 84 |
| 0.29 | 3.0 | 0.22 | 1.65 | 4.3 | 36 |
| 0.44 | 2.0 | 0.46 | 1.28 | 4.3 | 36 |
| 0.58 | 1.0 | 0.96 | 1.48 | 4.3 | 36 |
| 0.48 | 1.0 | 1.04 | 1.26 | 3.3 | 84 |
| 0.48 | 1.0 | 1.18 | 1.09 | 4.3 | 36 |
| 0.48 | 1.0 | 1.10 | 1.18 | 60.3 | 36 |

[a] Ratio of areas under bicarbonate and *N*-hydroxycarbamate peaks in the $^{13}C$ NMR spectrum.

Table II: Lags and Bursts in the Phosphorylation of Hydroxylamine at pH 9

| [hydroxylamine] (mM) | [bicarbonate] (mM) | rate constant for lag or burst ($min^{-1}$) obsd | rate constant for lag or burst ($min^{-1}$) calcd | component added last |
|---|---|---|---|---|
| 10 | 20 | 0.96 | 1.12 | bicarbonate |
| 10 | 30 | 1.20 | 1.12 | bicarbonate |
| 20 | 10 | 0.60 | 0.60 | bicarbonate |
| 20 | 20 | 0.40 | 0.60 | bicarbonate |
| 20 | 30 | 0.44 | 0.60 | bicarbonate |
| 30 | 10 | 0.44 | 0.40 | bicarbonate |
| 30 | 20 | 0.35 | 0.40 | bicarbonate |
| 30 | 30 | 0.34 | 0.40 | bicarbonate |
| 30 | 20 | 0.33 | 0.40 | hydroxylamine |
| 30 | 20 | 2.10 | 10.5 | bicarbonate plus carbonic anhydrase[a] |
| 20 | 20 | 0.28 | 0.60 | bicarbonate plus hydroxylamine[b] |
| 30 | 20 | 0.22 | 0.40 | bicarbonate plus hydroxylamine |

[a] 0.5 mg/mL carbonic anhydrase added at the same time as bicarbonate. [b] Bicarbonate and hydroxylamine preincubated at 375 mM concentration for 20 min prior to addition in 0.16 mL volume.

We can interpret these results in terms of the following equations:

$$HCO_3^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CO_2 \tag{4}$$

$$CO_2 + NH_2OH \underset{k_4}{\overset{k_3}{\rightleftharpoons}} HO\text{-}NH\text{-}COO^- \tag{5}$$

In these equations, $k_1$ and $k_3$ will be pH independent, while $k_2$ will increase and $k_4$ will decrease a factor of 10 per pH unit as the pH is increased. Since (1) the bicarbonate and hydroxylamine levels are essentially constant, because so small a proportion of them is converted to the carbamate, (2) the experiments were carried out at a low enough enzyme level so that carbamate was phosphorylated much more slowly than it was formed, and (3) the experiments were carried out below the $K_m$ of the carbamate so that the reaction rate was nearly proportional to the carbamate level present, the following equation applies:

$$\frac{dC}{dt} = \frac{k_1 B k_3 N - k_4 k_2 C}{k_2 + k_3 N} \tag{6}$$

where $B$, $C$, and $N$ are bicarbonate, *N*-hydroxycarbamate, and hydroxylamine concentrations, respectively. When integrated with the initial $C$ value equal to zero, eq 6 yields

$$C = \frac{k_1 k_3 BN}{k_2 k_4}[1 - e^{-k_2 k_4 t/(k_2 + k_3 N)}] \tag{7}$$

Since the lag experiments were carried out below the $K_m$ of the carbamate, the rate of product formation ($dP/dt$) is simply $V/K$ times $C$ as given by eq 7. Integration of this equation gives

$$P = (V/K)\frac{k_1 k_3 BN}{k_2 k_4}\left\{t - \frac{k_2 + k_3 N}{k_2 k_4}[1 - e^{-k_2 k_4 t/(k_2 + k_3 N)}]\right\} \tag{8}$$

as the time course of the reaction (the curve marked B or N in Figure 1). Equation 8 has an asymptote

$$P = (V/K)\frac{k_1 k_3 BN}{k_2 k_4}\left(t - \frac{k_2 + k_3 N}{k_2 k_4}\right) \tag{9}$$

The lag in this case is given by the intersection point of this asymptote on the time axis:

$$\text{lag} = \frac{k_2 + k_3 N}{k_2 k_4} \tag{10}$$

This equation predicts that the lag will increase linearly with hydroxylamine concentration but will be independent of bicarbonate concentration. The data in Table II (which are the reciprocals of the lag times from eq 10, that is, the actual rate constants for the exponential terms in eq 7 and 8) agree fairly well with this pattern. The rate constants $k_1$ and $k_2$ in eq 4 have values, at pH 9 and 25 °C, of $2.8 \times 10^{-4}$ $s^{-1}$ and 0.125 $s^{-1}$ (Gibbons & Edsall, 1963). The value of $k_3$ has been measured by Caplow (1968) at 10 °C as 104 $M^{-1}$ $s^{-1}$,[1] and

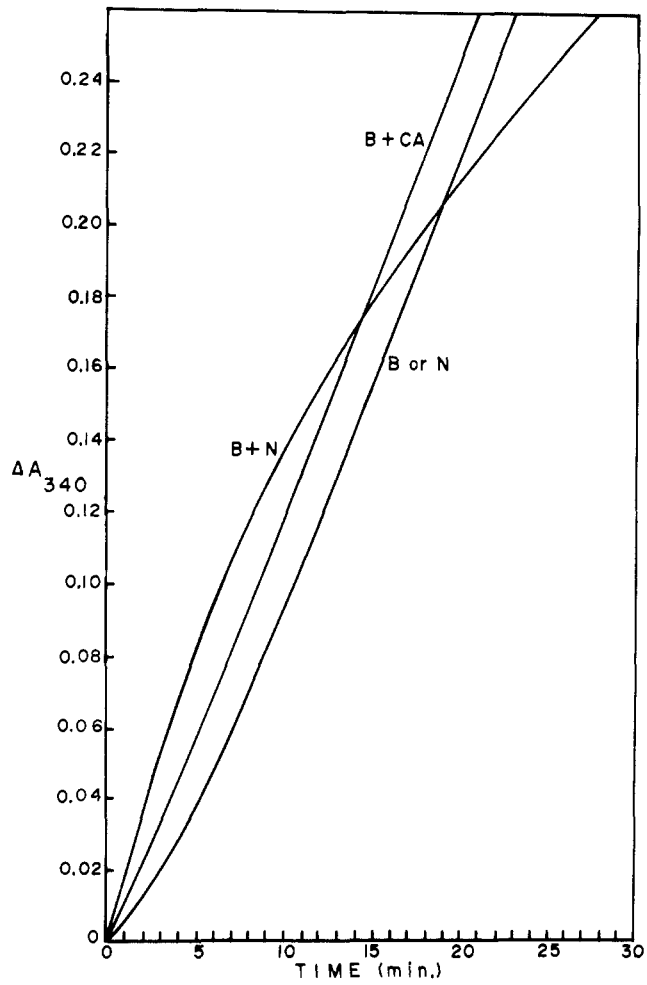



FIGURE 1: Time courses of the phosphorylation of hydroxylamine at pH 9 when either bicarbonate or hydroxylamine (B or N), bicarbonate and carbonic anhydrase (B + CA), or a mixture of bicarbonate and hydroxylamine (B + N) was added last to begin the reaction. The final concentrations were 30 mM hydroxylamine and 20 mM bicarbonate in each case.

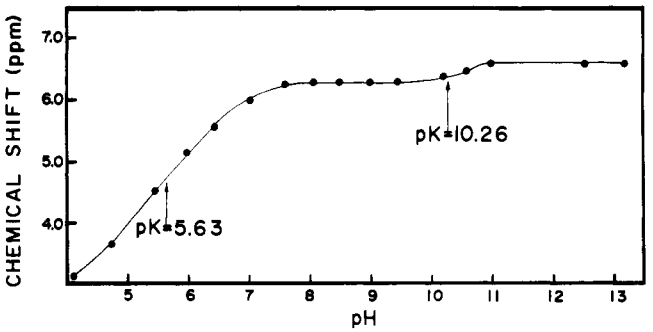



FIGURE 2: $^{31}P$ NMR chemical shift of *O*-phosphorylhydroxylamine as a function of pH. The values for the low-pH (zwitterion), medium-pH (monoanion), and high-pH (dianion) forms were 3.20 ± 0.11, 6.23 ± 0.05, and 6.60 ± 0.08 ppm, respectively.

thus $k_4$ has to be 0.175 $s^{-1}$ at pH 9. The lags calculated by eq 10 agree well with the experimental ones.

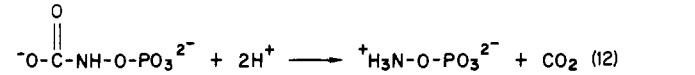
This model predicts that the rate constants for the bursts when both bicarbonate and hydroxylamine are added together should be the same as those seen in the lag experiments, since the differential equation for carbamate concentration is still given by eq 6.[2] The experimental values are about half of the predicted values, but this is because the initial carbamate levels were above the $K_m$, and thus the rate did not decrease proportionately to the concentration of *N*-hydroxycarbamate as it decomposed.

The addition of carbonic anhydrase eliminated most of the lag by establishing a rapid equilibrium between bicarbonate and $CO_2$. Since the level of $CO_2$ is now constant as a result of the rapid equilibrium, the equation for the formation of carbamate is now

$$dC/dt = k_3N[CO_2] - k_4C \qquad (11)$$

which when integrated gives $k_4$ as the rate constant for the lag (the same conclusion can be reached by letting $k_2$ greatly exceed $k_3N$ in eq 10). The observed lag was somewhat larger than this but probably includes some lag caused by the coupling system itself.

*Properties of the Reaction Product.* The product of the phosphorylation was identified by Cottam et al. (1968) as *O*-phosphorylhydroxylamine. This compound was first prepared by Jencks & Gilchrist (1965) by reaction of phosphoramidate and hydroxylamine at pH 7 and 39 °C for 40 min. As long as hydroxylamine was above 1 M, the major product was *O*-phosphorylhydroxylamine, but at lower hydroxylamine concentrations, considerable phosphate was formed. Jencks & Gilchrist (1965) proposed that both *N*- and *O*-phosphoryl products were formed but that the former was unstable and hydrolyzed unless it reacted first with hydroxylamine to form *O*-phosphorylhydroxylamine in a reaction analogous to that of phosphoramidate. *O*-Phosphorylhydroxylamine had a half-life in acid 53 times that of phosphoramidate and formed a phosphorylated oxime with furfural with the expected spectral properties (Jencks & Gilchrist, 1965). The enzymatic product of the pyruvate kinase reaction had the same properties (Cottam et al., 1968). It should also be noted that *N*-methyl- and *N*,*N*-dimethylhydroxylamines are phosphorylated by pyruvate kinase but *O*-methylhydroxylamine is not (Cottam et al., 1968). We have determined the p*K*s of *O*-phosphorylhydroxylamine by a $^{31}P$ NMR monitored pH titration (Figure 2). A p*K* of 5.63 ± 0.07 corresponding to a large (3 ppm) perturbation in chemical shift upon change in protonation state and a p*K* of 10.3 ± 0.5 corresponding to a smaller (0.4 ppm) perturbation were observed. These p*K*s are consistent with *O*-phosphorylhydroxylamine resulting from decarboxylation of the pyruvate kinase reaction product:[3]




The high and low p*K*s correspond to amine and phosphate ionizations, respectively. For comparison, the p*K*s of phosphoramidate and several amino phosphonates are listed in Table III. Note that while in hydroxylamine itself the p*K* of the amino group is lowered from the value of 9.3 for ammonia to 6 by the inductive effect of the hydroxyl group, the inductive effect of $-OPO_3^{2-}$ is similar to that of $-CH_2PO_3^{2-}$, and both are only slightly electron withdrawing, presumably because of the two negative charges. *O*-phosphorylhydroxylamine appears to be stable over the pH range covered in Figure 2, since samples from the pH extremes showed the expected chemical shifts when adjusted to neutral pH.

*Conclusions.* *N*-Hydroxycarbamate is an isosteric analogue of glycolate, and we presume that it is acting as such in the phosphorylation reaction, since glycolate is a good substrate for pyruvate kinase at high pH and the pH dependences of the glycolate- and bicarbonate-dependent hydroxylamine reactions are similar ($V/K$ values decreasing a factor of 10 per pH unit below a p*K* just above 9; Dougherty, 1982).

Table III: p*K* Values for *O*-Phosphorylhydroxylamine and Related Compounds[a]

| | p$K_2$ (phosphate) | p$K_3$ (amine) |
|---|---|---|
| $NH_3^+PO_3H^-$ | 3.0,[b] 3.6 | 8.2,[b] 8.16[c] |
| $NH_3^+OPO_3H^-$ | 5.63[d] | 10.3[d] |
| $NH_3^+CH_2PO_3H^-$ | 5.35 | 10.0 |
| $NH_3^+(CH_2)_2PO_3H^-$ | 7.00 | 10.8 |
| $NH_3^+(CH_2)_4PO_3H^-$ | 7.55 | 10.9 |
| $NH_3^+(CH_2)_5PO_3H^-$ | 7.65 | 11.0 |

[a] Values are from Chavane (1949), except as noted. [b] Meyerhof & Lohmann (1928). [c] Chambers & Khorana (1958). [d] Present work.

**Registry No.** Bicarbonate, 71-52-3; pyruvate kinase, 9001-59-6; hydroxylamine, 7803-49-8; *N*-hydroxycarbamate, 4464-01-1; *O*-phosphorylhydroxylamine, 20292-78-8.

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[1] This value will certainly be higher at 25 °C than at 10 °C, so that the calculated value of $k_4$ is too low. This makes little difference in the calculated lags, since $k_2$ is less than $k_3N$ in eq 8 at pH 9, and $k_3$ and $k_4$ will be in error by the same factor.

[2] When *C* is not zero at the beginning of the experiment, the exponential term in eq 7 is multiplied by $1 - C_0/C_\infty$ where $C_0$ and $C_\infty$ are the levels of carbamate at the beginning of the experiment and at final equilibrium (neglecting removal by the enzymatic phosphorylation, which removes only a very small amount of material during the time the reaction is followed). When $C_0$ exceeds $C_\infty$, as in the burst experiments where the level of carbamate is initially higher because bicarbonate and hydroxylamine have been preincubated in more concentrated solution and diluted into the reaction mixture, the level of *C* falls with time rather than increasing as in eq 6. The equation comparable to eq 8 has $1 - C_0/C_\infty$ multiplied times the $1 - \exp[-k_2k_4t/(k_2 + k_3N)]$ term, and in the equations comparable to eq 9 and 10, the same factor multiplies $(k_2 + k_3N)/(k_2k_4)$. Since this factor is negative in the burst experiments, the intersection point of the asymptote and the time axis is negative. The graphical method outlined under Data Analysis for determining half-times works equally well with bursts or lags and gives the rate constants for the exponential term directly.

[3] These results are not consistent with *N*-phosphorylhydroxylamine, since the p*K* of the hydroxyl group could not be as low as 10.3 (the value for hydroxylamine itself is 13.7, and *N*-phosphorylhydroxylamine would have two negative charges above pH 7) and since the $^{31}P$ NMR peak had a line width of <5 Hz and thus did not show the broadening expected by the quadrupolar nucleus of a nitrogen directly bonded to phosphorus.

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# Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Nucleotide Binding to the Chemically Modified Catalytic Subunit†

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ABSTRACT: 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA) inactivates the catalytic subunit of the adenosine cyclic 3′,5′-monophosphate dependent protein kinase isolated from bovine cardiac muscle by covalent modification of lysine-71, whereas 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) react with cysteines-199 and -343 to inactivate the enzyme. All three of these reagents have been postulated to modify residues at or near the active site of the catalytic subunit. ATP (2 mM) in the presence of excess $Mg^{2+}$ (10 mM) protects the enzyme against inactivation by these reagents. AMP did not afford any protection, but adenosine slightly decreased the rate of inactivation. The specific effects of covalent modification of lysine-71 and cysteines-199 and -343 on nucleotide binding were characterized by fluorescence-polarization titrations with *lin*-benzoadenine nucleotides as fluorescent ligands. *lin*-Benzoadenosine is a competitive inhibitor of the catalytic subunit with respect to ATP with a $K_i$ (38 $\mu$M) similar to the $K_i$ for adenosine (35 $\mu$M). This value agrees well with the $K_d$ (32 $\mu$M) for adenosine determined by fluorescence-polarization titrations. *lin*-Benzoadenosine 5′-diphosphate (*lin*-benzo-ADP) has been shown to be a competitive inhibitor with respect to ATP [Hartl, F. T., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 2347], and *lin*-benzoadenosine 5′-triphosphate (*lin*-benzo-ATP) is a substrate for the phosphotransferase activity of the protein kinase. Chemical modification by FSBA, NBD-Cl, or DTNB resulted in greater than 85% inhibition of phosphotransferase activity as well as complete inhibition of *lin*-benzo-ADP and *lin*-benzo-ATP binding in the presence of 10 mM $Mg^{2+}$. *lin*-Benzoadenosine, on the other hand, bound to the enzyme with the same $K_d$ and stoichiometry (1 mol/mol) as it did to the unmodified enzyme ($K_d$, 26–35 $\mu$M). While all effectively displaced *lin*-benzoadenosine bound to the unmodified catalytic subunit, AMP—but not MgATP or MgADP—displaced the fluorescent probe from enzyme modified with NBD-Cl, DTNB, or FSBA. The $K_d$ for AMP (804–856 $\mu$M), however, was 25% greater for the modified enzyme. These reagents, which are thought to modify residues that are at or near the active site of the catalytic subunit, inactivate the enzyme by inhibiting nucleotide binding. This effect involves the region on the C subunit complementary to the $\beta$- and $\gamma$-phosphates of the ATP molecule as compared to the region complementary to the $\alpha$-phosphate of the nucleotide binding portion of the C subunit.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] dependent protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) catalyze the phosphorylation of polypeptidic serine and threonine residues. The enzyme is composed of dissimilar regulatory and catalytic subunits. The complete amino acid sequences of the bovine cardiac muscle type II regulatory subunit (Takio et al., 1982) and catalytic subunit (Shoji et al., 1981, 1983) are known. The enzyme has several distinct functional sites that contribute to overall enzyme activity. These sites include an ATP binding site, a protein substrate binding site, and a site(s) for divalent cations. Each has been studied by a variety of approaches. Armstrong et al. (1979a), Granot et al. (1979, 1980), and Rosevear et al. (1983) have utilized nuclear magnetic resonance and electron spin resonance to study the interaction of nucleotides, synthetic peptides, and divalent cations with the catalytic subunit and to characterize the conformation of the bound nucleotide. The binding constants for a series of nucleotide analogues have been determined in an effort to characterize the ATP binding site of the catalytic subunit (Hoppe et al., 1977, 1978; Bhatnagar et al., 1983).

Possible amino acid residues important to enzyme function have been assessed by chemical modification studies. A variety of sulfhydryl-directed reagents (NBD-Cl, DTNB, and iodoacetic acid) inhibit the ATP:protein phosphotransferase activity of the catalytic subunit, suggesting that a cysteine residue is situated at or near the active site (Sugden et al., 1976; Bechtel

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; AMP-PNP, adenosine 5′-($\beta$,$\gamma$-imidotriphosphate).

0006-2960/84/0423-4350$01.50/0 