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INHIBITION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE BY STREPTONIGRIN*

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Abstract—Streptonigrin, an antibiotic with antineoplastic activity, inhibited rat liver phosphoenolpyruvate carboxykinase with an I_{50} of 0.3 μM when excess $FeCl_2$ was present. No inhibition occurred in the absence of added metal ion. Inhibition was partial and noncompetitive versus ITP and oxalacetic acid. The enzyme was more susceptible to inhibition by streptonigrin in the absence of substrates. Fe^{2+} supported inhibition by streptonigrin to a greater extent than did Fe^{3+}, while Mn^{2+} activated the enzyme in the presence of streptonigrin. For maximum inhibition, at least a 3-fold molar excess of iron over streptonigrin was required. The methyl ester of streptonigrin was also an inhibitor (I_{50} = 4 μM) while the fragment containing the C and D rings was not, indicating that inhibition did not depend solely on the presence of the picolinic acid moiety. When oxalacetate synthesis was measured, streptonigrin plus iron had no more effect on enzymatic activity than iron alone, and Mn^{2+} was capable of stimulating the streptonigrin–Fe^{2+} inhibited enzyme.

The antibiotic streptonigrin‡ is a powerful but extremely toxic antineoplastic agent [2–4]. Early studies of the compound indicated that it possessed activity against a variety of tumors, but the problems of toxicity were severe enough to curtail development of the drug.

Studies of the mechanism of the devastating effects of streptonigrin in the cell have been concerned with its role in nucleic acid metabolism [5], cell replication [6], and cell respiration and phosphorylation [1, 7]. To date, its potential activity as an effector of specific enzymes remains mostly unexplored. In this study, we describe the action of streptonigrin on the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK).§

Certain structural features of streptonigrin make it a likely candidate to modify the activity of PEPCK. The C ring of the molecule (Fig. 1) is a substituted picolinic acid. Several substituted picolinic acids can alter the activity of PEPCK in the presence of divalent transition metals [8, 9]. Of these, 3-mercaptopicolinic acid is the most effective inhibitor [10, 11], while the closely related compound 3-aminopicolinic acid permits activation of the enzyme by Fe^{2+} [12] in a manner that very strongly resembles that of the naturally occurring activator, ferroactivator protein [13]. All of the picolinic acid effectors of PEPCK require a divalent metal, optimally Fe^{2+}, to exert maximum effect [8, 9]. Interestingly, the same may be true for the cytotoxic effects of streptonigrin. Complexes of streptonigrin and divalent metal, as well as ternary complexes of streptonigrin, divalent metal, and DNA, have been observed [14–16]; the presence of metal has been reported to be a requirement for such complex formation [14]. In addition, the bacteriocidal activity of streptonigrin is enhanced in the presence of metals [15].

We can now expand the list of effects of streptonigrin at the molecular level to include metal-dependent inhibition of PEPCK. In the course of characterizing the inhibition, however, it became apparent that our working hypothesis—inhibition analogous to that of 3-mercaptopicolinic acid—could only partly explain the results, and that a more complex mechanism must be involved.

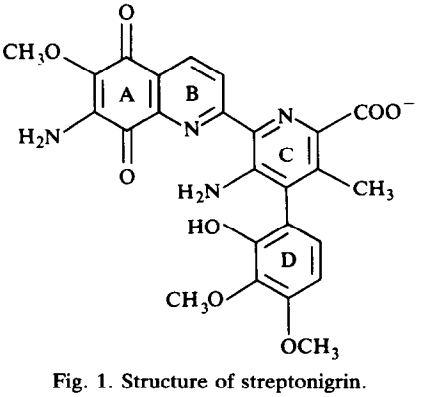

Fig. 1. Structure of streptonigrin.

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‡ The antibiotic valacidin, isolated independently by Dr. Robert Hamill at the Lilly Research Laboratories, was later found by him to be identical with streptonigrin. An early paper [1] describing the effect of this compound on tumor glycolysis and mitochondrial respiration used the name valacidin.

§ Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; and TEA, triethanolamine.

MATERIALS AND METHODS

Materials. Streptonigrin was provided by Dr. Robert Hamill, Lilly Research Laboratories, Indianapolis, IN; it was recrystallized several times. Streptonigrin methyl ester (NSC 45384) was the gift of Dr. John Douros, Natural Products Branch, Division of Cancer Treatment, NCI, Bethesda, MD. The C–D ring moiety was the gift of Dr. C. C. Cheng, Midwest Research Institute, Kansas City, MO. Quinoline dicarboxylic acid was synthesized by a published procedure [17] involving oxidation of acridine with permanganate.

NADH and the Na salts of ITP, IDP, and ADP were obtained from P-L Biochemicals, Milwaukee, WI; PEP, Hepes, TEA, OAA, DTT and superoxide dismutase from the Sigma Chemical Co., St. Louis, MO; and malate dehydrogenase, pyruvate kinase, lactate dehydrogenase, and glucose oxidase reagent from Boehringer-Mannheim, Indianapolis, IN. Tris was obtained from Schwarz-Mann, Orangeburg, NY. Inorganic chemicals were of the highest purity available.

Enzyme purification. Phosphoenolpyruvate carboxykinase was isolated from rat liver cytosol using the modifications of Colombo *et al.* [18] of the procedure of Philippidis *et al.* [19], with the following changes. After an initial fractionation in ammonium sulfate of 40–60% saturation, the active material was dialyzed against 50 mM Tris–Cl (pH 7), 1 mM EDTA, 1 mM DTT; the same buffer was used for Sephadex-G100 chromatography. The active pool from DEAE-cellulose chromatography was precipitated with ammonium sulfate (70% of saturation), taken up in 10 mM Tris–Cl (pH 7), 0.1 mM EDTA, 1 mM DTT, and dialyzed against this buffer before chromatography on hydroxylapatite. The purified enzyme was stored under N_2 as the ammonium sulfate precipitate of the hydroxylapatite pool. Prior to use, it was dialyzed extensively against 10 mM TEA–Cl (pH 7.5), 20% (v/v) glycerol, 2 mM DTT; in this form it was stable up to 2 months. Specific activity of the enzyme in the absence of effectors in these experiments was 8–12 μmoles PEP $\cdot$ min^{-1} $\cdot$ mg^{-1} at 25°.

PEPCK concentrations were determined by the absorbance at 280 nm, using $\varepsilon_{0.1\%} = 1.66$ [18].

Ferroactivator protein was isolated by the method of Bentle and Lardy [20].

The cytosolic fraction of rat liver was prepared from rats fasted for 24 hr and killed by decapitation; the livers were perfused with 0.9% NaCl, homogenized in 3 vol. of 10 mM TEA–Cl (pH 7.5), 0.25 M sucrose, 1 mM DTT, and centrifuged at 100,000 *g* for 1 hr.

Reagent preparation. Solutions of streptonigrin and related compounds were prepared just before use by dissolving the solid compound in dioxane and were diluted to the appropriate concentration with 5 mM Hepes–Na (pH 7.5). Comparable amounts of dioxane alone were without effect on PEPCK activity.

Solutions of $FeCl_2$ were prepared fresh for each experiment using degassed, N_2-saturated water.

Streptonigrin and blood glucose. Male albino rats (King Laboratories, Oregon, WI) weighing 250–300 g were fasted for 24 hr before intraperitoneal injection of 1 mg/kg streptonigrin (2 mg/ml in 0.9% NaCl plus NaOH to dissolve streptonigrin) or 0.9% NaCl. Blood samples were taken from the tail vein before injection and at 30 min and 1, 2, and 4 hr after, deproteinized with perchloric acid, and analyzed for glucose using glucose oxidase (Boehringer–Mannheim kit).

Measurement of PEPCK activity. PEPCK was routinely assayed using the point method of Seubert and Huth [21], with the modifications of Bentle and Lardy [22]. When continuous assays were needed, the assays for PEP synthesis and OAA synthesis described by Colombo *et al.* [18] were used.

Unless otherwise noted, streptonigrin and other compounds were tested for inhibitory effects on PEPCK following a short incubation with the enzyme and divalent metal. Enzyme (1.5 to 2.5 μg) was incubated on ice in 4 mM Hepes–Na, 0.8 mM DTT, 10 μM EDTA (pH 7.5), in a final volume of 0.2 ml, in the presence of various concentrations of streptonigrin or other compound. The divalent metal chloride (usually $FeCl_2$) was added to begin the incubation; after 10 min, an aliquot of the incubation was diluted 10-fold into the reaction mixture to begin the reaction.

Activity is reported as the average of two to four determinations.

RESULTS

When PEPCK was incubated with streptonigrin and $FeCl_2$ prior to assay, enzymatic activity was strongly inhibited. Several features of the inhibition are illustrated in Fig. 2. First, inhibition was absolutely dependent on the presence of metal. If iron was omitted, all concentrations of streptonigrin tried were without effect. At any fixed level of $FeCl_2$, the greatest inhibition was seen at low concentrations

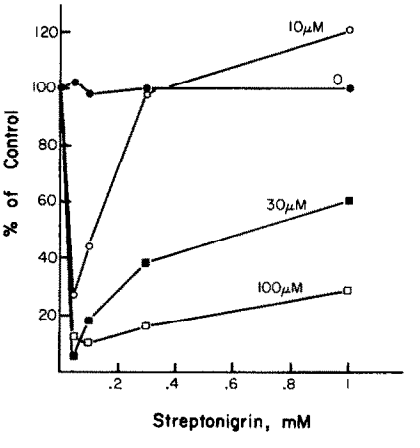

Fig. 2. Iron-dependent inhibition of PEPCK by streptonigrin. PEPCK (1.5 μg) was incubated in 0.15 ml volume in the presence of various concentrations of streptonigrin and $FeCl_2$ and assayed according to [18] in the presence of 2 mM ITP, 3 mM $MgCl_2$, and 1.5 mM OAA. Activity is expressed as percent activity in the absence of iron or streptonigrin; 100% = 8.9 μmoles PEP $\cdot$ min^{-1} $\cdot$ mg^{-1}. $FeCl_2$ was 0 μM, ●; 10 μM, ○; 30 μM, ■; 100 μM, □.

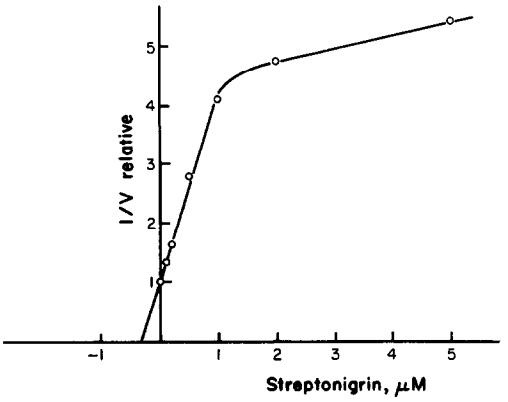

Fig. 3. I_{50} for streptonigrin inhibition. PEPCK (1.5 μg in 0.2 ml) was incubated with various concentrations of streptonigrin while $FeCl_2$ was held constant at 40 μM and assayed as in Fig. 2. Velocities are relative to enzyme activity in the presence of $FeCl_2$ and the absence of streptonigrin [12.0 μmoles·min^{-1}·(mg^{-1} protein)].

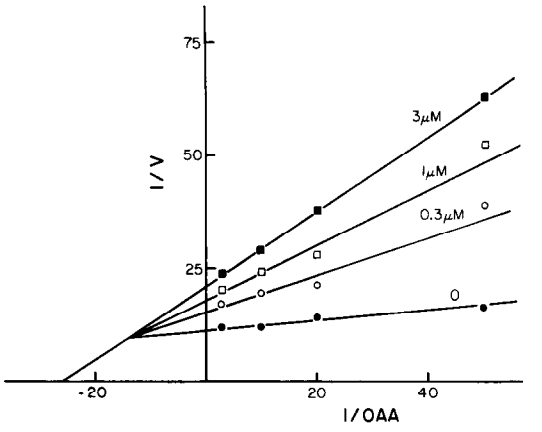

Fig. 4. Initial velocity pattern in the presence of streptonigrin. PEPCK (2.5 μg) was incubated in 0.1 ml final volume in the presence of various levels of streptonigrin and 40 μM $FeCl_2$ and, then, assayed continuously at various levels of OAA (expressed as mM) and at constant levels of 2 mM ITP and 4 mM $MgCl_2$. Velocities are expressed as ΔA_{340}/min and are corrected for non-enzymatic decarboxylation of OAA. Streptonigrin concentrations are designated in the figure.

of streptonigrin, and inhibition was actually relieved by higher amounts of the compound. Results were qualitatively similar if $FeCl_2$ concentrations were increased, and maximum inhibition was then observed at somewhat higher concentrations of streptonigrin.

An estimate of the concentration of streptonigrin necessary for 50% inhibition was obtained using a Dixon plot (Fig. 3). Inhibition was partial, with residual activities usually ranging from 5 to 20%. The initial portion of the plot, however, was linear, and extrapolated to a value of 0.3 μM for the I_{50}. For comparison, K_i values for 3-mercaptopicolinic acid have been reported in the range of 2–7 μM, depending on the purity of the enzyme preparation [9, 11].

The optimal conditions for inhibition involved a preliminary incubation of PEPCK, $FeCl_2$, and streptonigrin in the absence of substrates. Inhibition was also observed when enzyme that had been incubated with $FeCl_2$ was added to a mixture of reaction substrates and streptonigrin, but the I_{50} was greater (approximately 4 μM). The concentrations of streptonigrin reported in the figures and text are the concentrations in the preliminary incubation. Since the concentration of PEPCK in the preliminary incubation was usually 50–100 nM, 50% inhibition occurred in the presence of only a 3 to 6-fold molar excess of streptonigrin.

The nature of the inhibition was further explored using a continuous assay for PEP synthesis. Double-reciprocal plots of PEPCK activity as OAA was varied at fixed levels of streptonigrin (Fig. 4) revealed noncompetitive inhibition. The inhibition was hyperbolic, as indicated by its partial nature and confirmed by hyperbolic replots of slopes and intercepts versus streptonigrin concentration. Similar results were obtained when ITP was the variable substrate.

Varying the ratio of $FeCl_2$ to streptonigrin con-

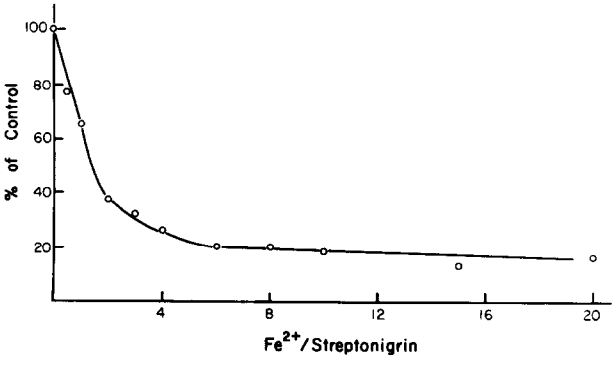

Fig. 5. Stoichiometry of iron requirement for streptonigrin inhibition. $FeCl_2$ was varied while streptonigrin was held constant (2 μM) in an incubation of 4 mM Hepes-Na (pH 7.5), 0.8 mM DTT, 5 μM EDTA, with 1.5 μg PEPCK per 0.2 ml. PEPCK was assayed as in Fig. 2. Free Fe^{2+} is assumed to be equal to [$FeCl_2$]–[EDTA]. One hundred percent activity = absence of $FeCl_2$, 5.6 μmoles PEP·min^{-1}·mg^{-1}.

Table 1. Specificity of metal ion requirement for inhibition of phosphoenolpyruvate carboxykinase by streptonigrin*

Additions	Relative PEPCK activity†	Ratio streptonigrin present/absent
None	100	
30 μM Fe^{2+}	136	0.36
+ 2 μM streptonigrin	49	
30 μM Fe^{3+}	97	0.61
+ 2 μM streptonigrin	59	
30 μM Mn^{2+}	305	0.83
+ 2 μM streptonigrin	254	
30 μM Zn^{2+}	106	0.89
+ 2 μM streptonigrin	94	

* PEPCK (1.5 μg) was preincubated for 5 minutes at 0°, with metal and streptonigrin, in 4 mM Hepes, 0.8 mM DTT, 5 μM EDTA (pH 7.5), prior to assay, and then assayed as in Fig. 2.

† Relative activity of 100 = 6.8 nmoles PEP/min.

firmed the requirement for an excess of iron. Figure 5 illustrates the optimal stoichiometry. Extrapolation of the early part of the curve suggests that a 3:1 complex, or at least a complex formed in the presence of a 3:1 molar ratio of iron to streptonigrin, is the inhibitory species. In practice, slightly higher ratios of iron to streptonigrin were required for maximum inhibition.

Several metals were tested for the ability to promote streptonigrin inhibition. In Table 1, their efficacies are compared. Only Fe^{2+} was clearly capable of generating an inhibitor. Since some Fe^{2+} could have been generated from Fe^{3+} under the conditions used, the inhibition in the presence of Fe^{3+} could easily have been due to Fe^{2+}. Mn^{2+}, a good activator of the native enzyme [22], was nearly as good an activator in the presence of streptonigrin. Zn^{2+} did not support the inhibition by streptonigrin.

As a working hypothesis, we had assumed that inhibition of PEPCK by streptonigrin was analogous to inhibition by 3-mercaptopicolinic acid and involved the picolinic acid portion of the molecule.

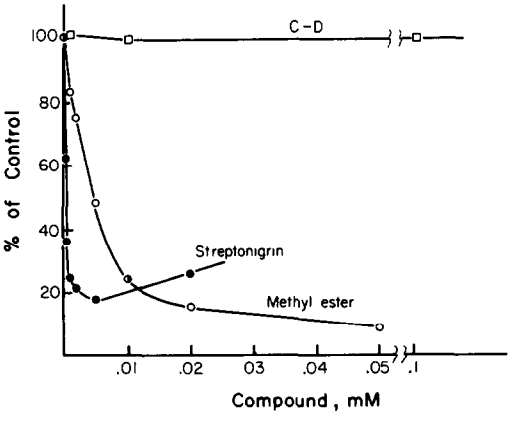

Fig. 6. Structural requirements for inhibition. Streptonigrin or the indicated compounds were incubated with 1.5 μg PEPCK in 0.2 ml, in the presence of 40 μM $FeCl_2$. PEPCK was assayed as in Fig. 2. One hundred percent activity is taken as activity in the absence of inhibitor (11 μmoles $PEP \cdot min^{-1} \cdot mg^{-1}$).

In order to determine the importance of the picolinic acid group for inhibition of PEPCK, we tested several derivatives of streptonigrin for effects on PEPCK. Their behavior was inconsistent with this hypothesis.

In Fig. 6, the behavior of streptonigrin is compared to that of two related compounds. Previous studies of other picolinic acids indicated that esterification of the carboxyl function led to a loss of effectiveness against PEPCK [9]. The methyl ester of streptonigrin, however, was a good inhibitor of PEPCK, with an I_{50} of about 4 μM. Like streptonigrin, the methyl ester was ineffective in the absence of iron. In contrast, a fragment consisting of the C and D rings of streptonigrin [23] was not inhibitory in the presence or absence of iron, at concentrations up to 1 mM. Thus, it appears that some structural feature of the compound other than the picolinate ring is necessary to produce the observed inhibition.

Although not an inhibitor itself, the C–D moiety was capable of competing with streptonigrin to relieve inhibition by the latter in the presence of iron. Figure 7 illustrates this effect. Competition could be detected when the concentration of C–D moiety was comparable to or greater than the free Fe^{2+} concentration.

Unlike the picolinic acid inhibitors of PEPCK, streptonigrin was less effective when added directly to the reaction mixture than when incubated with the enzyme and iron in the absence of substrates. In this respect it is similar to quinoline-2,3-dicarboxylic acid, an analog of quinolinic acid. When quinoline dicarboxylic acid is incubated with pure enzyme and 30 μM iron and subsequently assayed, the compound is an inhibitor with an I_{50} of about 0.4 mM [24]. When it was included in the reaction mixture, however, it had only a slight effect on iron-activated enzyme. The common structural feature of quinoline dicarboxylic acid and streptonigrin is greater bulk than the picolinic acid derivatives. The presence of extra rings apparently does not prevent interaction with the enzyme in the presence of metal but no substrates, but it may be enough to hinder binding of the inhibitor when substrates are bound.

It is unlikely that streptonigrin works as an inhib-

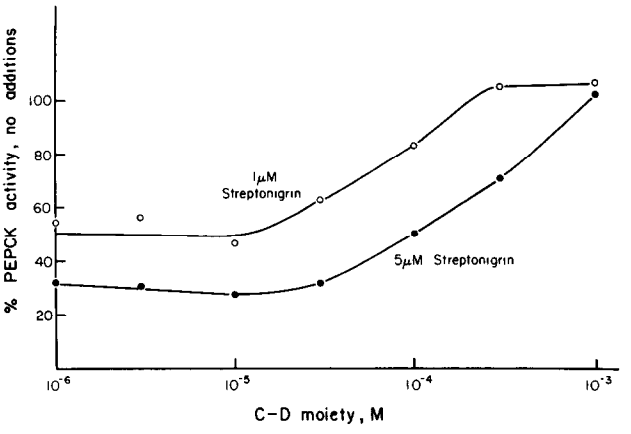

Fig. 7. Competition by CD fragment. Streptonigrin was held constant at 1 μM (○) or 5 μM (●) in the standard incubation with 40 μM $FeCl_2$; the concentration of CD fragment was varied. PEPCK was assayed as in Fig. 2. Activity of 100% corresponds to activity in the absence of streptonigrin, 8.3 μmoles $PEP \cdot min^{-1} \cdot mg^{-1}$.

itor of PEPCK at low doses *in vivo*. Intraperitoneal administration of 1 mg/kg streptonigrin to 24-hr fasted rats did not produce a significant effect on blood glucose up to 4 hr after injection. Higher doses have been reported to be toxic and, therefore, were not used. Also, much higher doses were required for inhibition of PEPCK to be manifested when liver cytosol was used in place of purified PEPCK. This was not the result of the presence of ferroactivator, as inhibition was still observed when purified ferroactivator was included in an incubation containing purified enzyme, iron, and streptonigrin (data not shown).

The effects of streptonigrin on the synthesis of OAA differed from its effects on PEP synthesis. The two reactions catalyzed by PEPCK have different dependencies on divalent metal; PEP synthesis is stimulated 3 to 4-fold by Mn^{2+} or Fe^{2+} [8, 25, 26], while OAA synthesis has an absolute requirement for a divalent transition metal [27]. Both reactions require a metal (Mg^{2+} or Mn^{2+}) to bind the nucleotide [28]. Normally, therefore, OAA synthesis is measured in the presence of sufficient Mn^{2+} to fill both the nucleotide and transition metal sites. When Mn^{2+} was the sole metal provided in the reaction mixture, PEPCK that had been incubated with $FeCl_2$ and streptonigrin catalyzed OAA synthesis at rates the same or slightly higher than rates observed when the enzyme had been incubated with $FeCl_2$ alone (Table 2). When assay conditions were modified so that Mg^{2+} was the only metal available in the reaction mixture, PEPCK that had been incubated with $FeCl_2$, or $FeCl_2$ and streptonigrin, showed a burst of activity when added to the reaction mix that quickly decayed to about 5% of the rate in the presence of Mn^{2+}. This low level of reaction was probably the result of the small (< 3 μM) amounts of $FeCl_2$ carried over from the preliminary incubation.

In order to assess the ability of Mn^{2+} to reverse inhibition of PEPCK by streptonigrin plus Fe^{2+}, we measured PEPCK activity using a continuous assay [18] in the presence of the standard reaction mixture (4 mM $MgCl_2$) and one that had been supplemented with 0.2 mM $MnCl_2$. When the enzyme was incubated with $FeCl_2$ in the presence or absence of streptonigrin, the rate achieved in the presence of Mn^{2+} was never more than 50% of the rate observed for enzyme that had not been incubated with $FeCl_2$

Table 2. Effect of streptonigrin in the "reverse" direction*

Condition	Activity† (nmoles/min)
Control	15.3
+ 30 μM Fe^{2+}	5.8
+ 30 μM Fe^{2+}, 0.2 μM streptonigrin	6.4
+ 30 μM Fe^{2+}, 1 μM streptonigrin	6.5
+ 30 μM Fe^{2+}, 5 μM streptonigrin	6.7

* "Reverse" direction: PEP + IDP + $CO_2 \rightarrow$ OAA + ITP.

† Assayed according to [18] in the presence of 2 mM $MnCl_2$. Pure PEPCK (2.1 μg) was incubated with $FeCl_2$ and streptonigrin as indicated, in a final volume of 0.1 ml. An aliquot of 0.09 ml was added to the reaction cuvette to initiate the assay.

Table 3. Activation of streptonigrin-inhibited enzyme by Mn^{2+}

Condition	Activity* (nmoles/min) in presence of: Mg^{2+}	Mg^{2+} + Mn^{2+}
Control	15	60
+ 30 μM Fe^{2+}	15	32
+ 30 μM Fe^{2+} + 1 μM streptonigrin	10	31
+ 30 μM Fe^{2+} + 5 μM streptonigrin	8.3	24

* PEPCK (2.3 μg) was incubated in 0.1 ml total volume with $FeCl_2$ and streptonigrin as indicated; 0.09 ml was used to initiate reaction. Assay was a continuous assay for PEP synthesis [18]. $MgCl_2$ was always 4 mM in the reaction mixture; when added, $MnCl_2$ was 0.2 mM. ITP (2 mM) and OAA (0.3 mM) were present in all assays.

(Table 3). However, in the presence or absence of streptonigrin, activities of Fe^{2+}-treated samples were increased 2 to 3-fold in the presence of Mn^{2+}, and enzyme that had been inhibited by streptonigrin and Fe^{2+} was activated slightly more by Mn^{2+} than the Fe^{2+}-treated enzyme.

DISCUSSION

The activity of PEPCK was extremely sensitive to inhibition by streptonigrin. In the presence of 30 μM $FeCl_2$, 50% inhibition occurred at a streptonigrin concentration of 0.3 μM, compared to 7 μM for 3-mercaptopicolinic acid [9] under similar conditions. Inhibition by streptonigrin was detectable when the compound and PEPCK were present in roughly equimolar amounts. However, a comparison of streptonigrin with model compounds indicated that an intact picolinic acid system alone is neither sufficient for inhibition nor required.

At least three Fe^{2+} per streptonigrin molecule were required for maximum inhibition of PEPCK. This stoichiometry is similar to that observed by Rao [14] for the formation of complexes between DNA, streptonigrin, and Zn^{2+}, where 6–7 Zn^{2+} per streptonigrin gave maximum binding to DNA. The requirement for an excess of Fe^{2+} over streptonigrin is illustrated by the relief of inhibition that was observed when streptonigrin concentration was elevated above $FeCl_2$ concentration, even in the presence of 100 μM $FeCl_2$. This result implies that either the inhibitory species is not formed in appreciable amounts unless excess $FeCl_2$ is present, or that some non-inhibitory species prevents inhibition by competing with the inhibitory species, either for Fe^{2+} or for a site on the enzyme.

The streptonigrin molecule contains several possible sites for interaction with a metal in addition to the picolinic acid [14, 16]. The vastly different spectra and susceptibility to reduction of Cu^{2+}– and Zn^{2+}–streptonigrin complexes have led to a proposal that the two metals form structurally different complexes involving the B and C rings, with Cu^{2+} coordinated to the amine nitrogen of the C ring and quinoline nitrogen in the B ring, but Zn^{2+} coordinated to the ring nitrogens of the B and C rings as well as to the carboxyl group of the C ring [16]. Another system that could potentially bind metal is the aminoquinone of the A ring [14]. Thus, the binding of more than one Fe^{2+} per streptonigrin molecule is possible.

Another aspect of the molecular action of streptonigrin with possible relevance to inhibition of PEPCK is the chemistry of the quinone system. If the quinone is blocked to prevent reduction, its biological activity is lost [29]. Upon reduction, the compound can react with O_2 to generate superoxide anion. The production of O_2^- has been linked both to its lethality against bacteria [30] and its ability to cause breaks in DNA [31]. Superoxide production requires the presence of oxygen and reducing equivalents [30–32]; it is enhanced be Fe^{2+} [31]. The inhibition of PEPCK could be the result of generation of O_2^- or another reactive oxygen species that attacks the active site of the enzyme. A final possibility is a direct reaction of the quinone system with reactive thiols on the enzyme [33] to produce disulfides or streptonigrin adducts.

As a protection against loss of reactive thiols on the enzyme, dithiothreitol was always present. Superoxide dismutase, which decomposes O_2^- to H_2O_2 and O_2, had no effect on inhibition by streptonigrin. The inclusion of 50 mM ethanol as a scavenger of ·OH [34] was also incapable of preventing the inhibition. Thus, if a reactive oxygen species is responsible for the inhibition, it must be generated in close proximity to the site to be attacked.

Attempts to establish the reversibility of inhibition by streptonigrin were hampered by the rapid loss of activity observed in the presence of Fe^{2+}, whether streptonigrin was present or not. However, several results are consistent with reversible inhibition by streptonigrin. First, inhibition by a given level of streptonigrin did not increase with increasing length of incubation; instead, a maximum was reached by 1 min that was approximately constant for at least 15 min. In addition, covalent modification of the enzyme that resulted in loss of activity would be expected to cause inhibition of the reaction in both directions. In fact, no difference was discernible in the rates of synthesis of OAA between enzyme that had been incubated with $FeCl_2$ and streptonigrin, and enzyme incubated with $FeCl_2$ alone. The difference between the two directions is a strong argument in favor of a reversible mechanism.

A mechanism for inhibition of phosphoenolpyruvate carboxykinase by streptonigrin must take all of the above information into account. One scheme which fits the observations has the following steps. First, a complex involving at least two Fe^{2+} per molecule of streptonigrin forms. This complex has affinity for the transition metal site of phosphoenolpyruvate carboxykinase because of the picolinic acid function. Once directed to the active site of the enzyme by the metal—picolinate, the complex behaves as an inhibitor because of some effect of the A–B ring system (and probably another Fe^{2+}). This inhibition could be an inactivation caused by a reaction of the quinone, or by the generation of a toxic oxygen species at the active site, but is probably reversible.

REFERENCES

1. H. A. Lardy, D. Johnson and W. C. McMurray, *Archs Biochem. Biophys.* **78**, 587 (1958).
2. J. J. Oleson, L. A. Calderella, K. J. Mjos, A. R. Reith, R. S. Thies and I. Toplin, *Antibiot. Chemother.* **11**, 158 (1961).
3. H. C. Reilly and K. Sigiura, *Antibiot. Chemother.* **11**, 174 (1961).
4. D. T. Kaung, R. M. Wittington, H. Spencer and M. E. Patno, *Cancer* **23**, 1280 (1969).
5. M. A. Chirigis, J. W. Pearson, T. S. Papas, W. A. Woods, H. B. Wood Jr. and G. Spahn, *Cancer Chemother. Rep.* **57**, 305 (1973).
6. N. S. Mizuno, *Biochim. biophys. Acta* **108**, 394 (1965).
7. P. Hochstein, J. Laszlo and D. Miller, *Biochem. biophys. Res. Commun.* **19**, 289 (1965).
8. R. E. Snoke, J. B. Johnston and H. A. Lardy, *Eur. J. Biochem.* **21**, 342 (1971).
9. M. J. MacDonald and H. A. Lardy, *Bioorg. Chem.* **7**, 251 (1978).

10. V. Kostos, N. W. DiTullio, J. Rush, L. Cieslinski and H. L. Saunders, *Archs Biochem. Biophys.* **171**, 459 (1975).
11. M. Jomain-Baum, V. L. Schramm and R. W. Hanson, *J. biol. Chem.* **251**, 37 (1976).
12. M. J. MacDonald and H. A. Lardy, *J. biol. Chem.* **253**, 2300 (1978).
13. L. A. Bentle, R. E. Snoke and H. A. Lardy, *J. biol. Chem.* **251**, 2922 (1976).
14. K. V. Rao, *J. pharm. Sci.* **68**, 853 (1979).
15. J. R. White, *Biochem. biophys. Res. Commun.* **77**, 387 (1977).
16. J. Hadju and E. C. Armstrong, *J. Am. chem. Soc.* **103**, 232 (1981).
17. C. Graebe and H. Caro, *Ber. dt. chem. Ges.* **13**, 99 (1880).
18. G. Colombo, G. M. Carlson and H. A. Lardy, *Biochemistry* **17**, 5321 (1978).
19. H. Philippidis, R. W. Hanson, L. Reshef, M. F. Hopgood and F. J. Ballard, *Biochem. J.* **126**, 1127 (1972).
20. L. A. Bentle and H. A. Lardy, *J. biol. Chem.* **252**, 1431 (1977).
21. W. Seubert and W. Huth, *Biochem. Z.* **343**, 176 (1965).
22. L. A. Bentle and H. A. Lardy, *J. biol. Chem.* **251**, 2916 (1976).
23. T. K. Liao, P. J. Wittek and C. C. Cheng, *Heterocyclic Chem.* **13**, 1283 (1976).
24. H. A. Lardy, M. L. Merryfield and M. J. MacDonald, in *Biochemical and Medical Aspects of Tryptophan Metabolism*, (Eds. O. Hayaishi, Y. Ishimura, and R. Kido), pp. 199–206. Elsevier, New York (1980).
25. D. D. Holten and R. C. Nordlie, *Biochemistry* **4**, 723 (1965).
26. D. O. Foster, H. A. Lardy, P. D. Ray and J. B. Johnston, *Biochemistry* **6**, 2120 (1967).
27. G. Colombo, G. M. Carlson and H. A. Lardy, *Biochemistry* **20**, 2749 (1981).
28. M. F. Utter and H. M. Kolenbrander, in *The Enzymes* (Ed. P. D. Boyer), 3rd Edn, Vol. VI, pp. 117–68. Academic Press, New York (1972).
29. S. Chaube, F. R. Kuffer and M. L. Murphy, *Cancer Chemother. Rep.* (part I), **53**, 23 (1969).
30. E. M. Gregory and I. Fridovich, *J. Bact.* **114**, 1193 (1973).
31. J. W. Lown and S-K. Sim, *Can. J. Biochem.* **54**, 219 (1976).
32. H. L. White and J. R. White, *Molec. Pharmac.* **4**, 549 (1968).
33. T. Posner, *Ann. Chem.* **336**, 85 (1904).
34. C. Beauchamp and I. Fridovich, *J. biol. Chem.* **245**, 4641 (1970).