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ADENYLOSUCCINATE SYNTHETASE AND ADENYLOSUCCINATE LYASE FROM *TRYPANOSOMA CRUZI*

SPECIFICITY STUDIES WITH POTENTIAL CHEMOTHERAPEUTIC AGENTS

THOMAS SPECTOR,*† RANDOLPH L. BERENS‡§ and J. JOSEPH MARR‡§
*Wellcome Research Laboratories, Research Triangle Park, NC 27709, U.S.A.
and
‡Departments of Medicine and Microbiology, St. Louis University School of Medicine, St. Louis, MO 63104, U.S.A.

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Abstract—Adenylosuccinate (succino-AMP) synthetase and succino-AMP lyase were isolated from epimastigotes of *Trypanosoma cruzi* by chromatography on phosphocellulose. The synthetase was capable of catalyzing the condensation of aspartic acid with IMP and several IMP analogs. The reaction with allopurinol ribonucleotide is of potential chemotherapeutic interest. This analog was slowly converted to its corresponding succino-AMP analog with a K_m' of 140 μM (cf. IMP at 10 μM) and a V_{max}' of 0.3 per cent the rate with IMP. The comparable reaction with this analog does not occur with succino-AMP synthetase from a representative mammalian source [T. Spector and R. L. Miller, *Biochim. biophys. Acta* **455**, 509 (1976)].

The protozoal succino-AMP lyase had a broad substrate specificity which was characteristic of this enzyme from many sources. It catalyzed the rapid and efficient cleavage of all the succino-AMP analogs that were produced by succino-AMP synthetase. Thus, these two enzymes appear to be responsible for the selective amination of allopurinol ribonucleotide in *T. cruzi*. The metabolically produced AMP analog may be the agent or a precursor of the agent that accounts for the anti-growth activity of allopurinol in these organisms. Similar selective amination was observed previously with these enzymes from *Leishmania donovani* [T. Spector, T. E. Jones and G. B. Elion, *J. biol. Chem.* **254**, 8422 (1979)]. Thiopurinol ribonucleotide was not a substrate of succino-AMP synthetase from *T. cruzi*, but it was an inhibitor with a $K_i = 33$ μM. Therefore, the weakness of thiopurinol's anti-growth activity with *T. cruzi* is not due to its inability to inhibit this enzyme.

Allopurinol (HPP, 4-hydroxypyrazolo[3,4-*d*]-pyrimidine)‖ and its riboside (HPPR) are effective growth inhibitors *in vitro* of *Leishmania donovani*, *L. braziliensis* and *L. mexicana* [1–3]. Both compounds are metabolically converted to the ribonucleoside 5′-monophosphate, HPPR-MP, which, in turn, is aminated to form 4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleotide (APPR-MP). This AMP-analog is then converted to the triphosphate and incorporated into RNA [3–5]. A study of succino-AMP synthetase, EC 6.3.4.4, and succino-AMP lyase, EC 4.3.2.2 (see Fig. 1 for the reactions) from *L. donovani* [6] revealed that, compared to the mammalian counterpart [7], the protozoal synthetase has a slightly different substrate specificity which enables it to convert HPPR-MP to succino-APPR-MP. The protozoal lyase with its typical broad specificity rapidly converted this intermediate to APPR-MP. Since all of these leishmanial parasites form APP-ribonucleotides [3–5], it was suggested [6] that this unusual succino-AMP synthetase may be common to the order Kinetoplastida and could account for their selective sensitivity to HPP and HPPR.

Tyrpanosoma cruzi, the causative organism for Chagas disease in humans, is another kinetoplastid flagellate that is suceptible to inhibition by HPP from which it also forms APP ribonucleotides [8]. However, it differs from the *Leishmania* organisms because it is not inhibited by HPPR [8]. Furthermore, thiopurinol (TPP), the 4-thio analog of HPP, which is also active against leishmania [9] has only weak activity against *T. cruzi*¶. In leishmania, TPP is metabolized to the ribonucleoside 5′-monophosphate, TPPR-MP, which is a good inhibitor but not a substrate of succino-AMP synthetase from *L. donovani* [9]. Since inhibition of succino-AMP synthetase is one presumed locus of action of TPPR-MP in leishmania [9], the poor inhibitory effect of TPP

† Author to whom all correspondence should be addressed.

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‖ Abbreviations: HPP, allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine; TPP, thiopurinol (4-thiopyrazolo[3,4-*d*]pyrimidine); APP, 4-aminopyrazolo[3,4-*d*]-pyrimidine; succino-AMP, adenylosuccinate; ara, β-D-arabinofuranosyl; R, ribonucleoside; MP, monophosphate; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

¶ R. L. Berens and J. J. Marr, unpublished data.

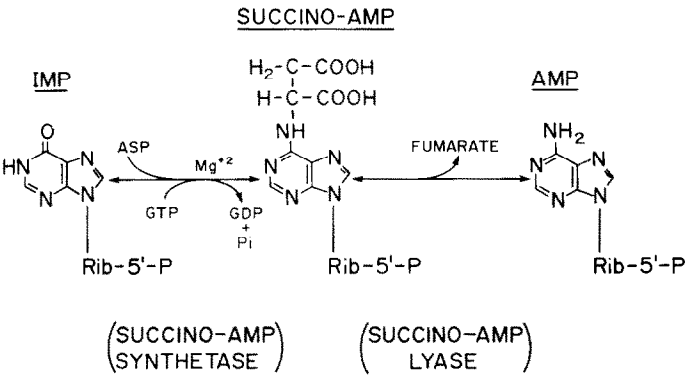

Fig. 1. Enzymes responsible for the metabolic conversion of IMP to AMP.

against *T. cruzi* suggested the possibility that succino-AMP synthetase might be different in the two genera of protozoa.

It, therefore, became of interest to verify the existence of and characterize succino-AMP synthetase and succino-AMP lyase from *T. cruzi*. The purification and substrate specificity of these two enzymes are reported here.

MATERIALS AND METHODS

Chemicals

References for the purchase or synthesis of all chemicals except TPPR-MP are presented elsewhere [6]. TPPR-MP was synthesized at the Wellcome Laboratories by W. H. Miller. It was >99 per cent homogeneous as judged by high performance liquid chromatographic (h.p.l.c.) analysis.

Succino-AMP synthetase assays

The spectrophotometric assay (Assay I) that couples the formation of GDP to NADH reduction was monitored at 340 nm [6, 7]. The direct spectrophotometric assays were monitored at 280 nm (Assay IIa) [6], or, to avoid the high absorbance of the concentrated substrates, at 283 nm (Assay IIb), $E = 8.4\ mM^{-1}$. Radiochemical assays that used purified [^{14}C]aspartic acid and high voltage electrophoresis (Assay III) were performed as previously described [6] except that the [^{14}C]aspartic acid was purified by electrophoresis for 1 hr instead of 3 hr. The shorter time of electrophoresis resulted in improved blank rates (IMP or enzyme omitted) with < 0.02 per cent of the counts appearing in the succino-AMP spot and < 0.01 per cent in the 8-aza-succino-AMP spot. Unless otherwise stated, all reagents were used at their standard concentrations which were previously reported [6]. Similar velocities were obtained with all of these assay methods.

Production of T. cruzi *epimastigotes*

These organisms were cultured as previously described [10].

Purification of succino-AMP synthetase and succino-AMP lyase

The first part of this procedure was done in biohazard containment facilities. *T. cruzi* epimastigotes (1.9×10^{11} cells) were quick-frozen, thawed at 5°, and sonicated for 30 sec. All steps were carried out at 0–5°. The sonicate was centrifuged at 49,000 *g* for 30 min and the 20 ml supernatant fraction was mixed with 80 ml of Buffer A [5 mM sodium phosphate (pH 6.6), 0.2 mM $MgCl_2$]. As a safety precaution, this solution was recentrifuged. The resultant supernatant fraction was applied onto a 2.2×5.5 cm column containing phosphocellulose gel [6]. The column was washed with 50 ml of Buffer A and stored for 24 hr at 5°.

The column was then washed with 50 ml of Buffer A containing 70 mM KCl followed by 10 ml of Buffer A. The enzymes were eluted with an 80 ml linear gradient of Buffer A vs 190 mM sodium phosphate (pH 6.6), 0.2 mM $MgCl_2$ at a flow rate of 0.85 ml/min and 5 min/fraction. Succino-AMP lyase was eluted in a sharp peak at about 115 mM phosphate. Approximately 4000 units (1 unit is that amount of enzyme that produces 1 nmole AMP/min at 30°) with specific activity of 450–650 units/mg were collected. The enzyme was stored at 4° in 1 mM EDTA and 0.002% NaN_3. The phosphate concentration was decreased immediately before use by filtration on a calibrated Sephadex G-25 column equilibrated in 20 mM sodium phosphate (pH 6.6), 1 mM EDTA and 0.002% NaN_3.

Succino-AMP synthetase was eluted in a sharp peak at about 150 mM phosphate. Approximately 750 units of 100–150 units/mg were collected, divided into 1 ml aliquots, quick-frozen, and stored at −80°.

All other assays, conditions and methods are described elsewhere [5].

RESULTS

Succino-AMP synthetase

General properties. Succino-AMP synthetase was reasonably stable to storage at −80°, loosing < 15 per cent activity during 3 weeks. It was also completely stable for at least 15 min when incubated at 30° in the presence of 150 mM phosphate. This feature was advantageous because it permitted a 2-min incubation at 30° with 100 mM $MgCl_2$ which completely inactivated the traces of succino-AMP lyase

(see below) that were present in the synthetase fractions. This incubation, followed by desalting on a calibrated Sephadex G-25 column in 20 mM HEPES buffer (pH 7.0), 0.2 mM $MgCl_2$, was routinely performed immediately prior to studies which used either Assay IIa, IIb or III which directly measure succino-AMP formation. It was not necessary for Assay I which indirectly measures the formation of GDP. The enzyme was also stable during the reactions with the slower substrates that required incubations up to 4 hr.

The preparation was essentially free of nucleotide cleaving activity (< 0.03 per cent of the succino-AMP forming activity) when measured with 0.1 mM IMP as the representative substrate. Assay I, the coupled assay, revealed a 4–5 per cent blank rate (IMP omitted) which was attributed to GTPase and/or NADH oxidase activity.

Reaction velocities increased linearly with the concentration of succino-AMP synthetase when measured over the range from 0 to 4 units/ml.

Substrate specificity. The kinetic constants for IMP and its analogs are presented in Table 1. The slow blank rate observed with the coupled assay (see above) became more significant relative to the slower substrates. Therefore, 2′-dIMP and ara-IMP were evaluated by the direct spectral assay at 283 nm (Assay IIb). Nevertheless, a small, but relatively significant, blank rate (1.7 per cent of the rate of the standard assay with IMP) was still present and was responsible for the rather high standard errors of the K_m' determinations for these substrates. The products of all substrates were detected from reactions with [^{14}C]aspartic acid as the common substrate (Assay III). These reactions with 1 mM substrate were analyzed for the formation of ^{14}C-labeled product at 0, 1 and 2 or 0, 2 and 4 hr [6]. The rates of product formation were linear with respect to time for all the substrates.

Succino-AMP lyase

General properties. Succino-AMP lyase slowly lost its activity during storage at 4° with a half-life of 20–25 days. It was completely inactivated within 2 min when incubated at 30° in the absence of protecting agents. Considerable protection was afforded by 100 mM phosphate; ≥ 1 μM succino-AMP, 2′-deoxysuccino-AMP or ara-succino-AMP; ≥ 10 μM succino-APPR-MP; ≥ 20 μM 8-aza-succino-AMP. The enzyme could also be inactivated within 2 min at 30° in the presence of 150 mM phosphate if 100 mM $MgCl_2$ were present.

The preparation was found to be free of nucleotide cleaving activity (< 0.005 per cent of the succino-AMP cleaving activity) when assayed with 0.1 mM IMP.

Reaction velocities were measured during the linear portion of the reaction, that is, before there was significant depletion of substrate or significant inactivation of enzyme. The rates were linear with respect to the concentration of succino-AMP synthetase when measured over the range from 0 to 8 units/ml.

Substrate specificity. Succino-AMP lyase from *T. cruzi* catalyzed the rapid cleavage of all the succino-AMP analogs produced by the preceding reaction with succino-AMP synthetase. The K_m and V_{max} values are presented in Table 2. The range of the substrate concentration was limited to ≥ 10 μM for succino-APPR-MP and ≥ 20 μM for 8-aza-succino-AMP because of their inability at lower concentrations to adequately protect the enzyme against inactivation at 30°. The double-reciprocal plots of the data are shown in Fig. 2.

Product formation was confirmed by cochromatography of the reaction product with the appropriate authentic compound (see Table 2) as previously described [6]. Reactions with 1 mM substrate were incubated for 48 hr at 23°. All reactions went to > 85 per cent completion.

DISCUSSION

The above studies indicate that the enzymes involved in the metabolic conversion of IMP to AMP in *T. cruzi* are markedly similar to those of *L. donovani*. The substrate specificities of succino-AMP synthetases from these two organisms were essentially identical but were clearly distinguishable from that of the mammalian enzyme. By virtue of a slight difference in the enzyme of the parasites, HPPR-MP could be converted to succino-APPR-MP in these organisms, but not in the host. A representative mammalian succino-AMP synthetase could not catalyze the condensation of aspartic acid with either HPPR-MP or 8-aza-IMP [7].

Succino-AMP lyase from *T. cruzi* has the broad

Table 1. Substrate specificity of succino-AMP synthetase from *T. cruzi*

Compound	K'_m ± S.E. (μM)	V_{max} (relative)	Product
IMP	9.8 ± 0.6*	100	Succino-AMP
2′-dIMP	450 ± 210†	13‡	2′-Deoxy-succino-AMP
Ara-AMP	200 ± 100†	9‡	Ara-succino-AMP
8-Aza-IMP	93 ± 9§	0.7‖	8-Aza-succino-AMP
HPPR-MP	143 ± 12§	0.3‖	Succino-APPR-MP
TPPR-MP	33 ± 5§	< 0.05‖	

* The coupled-spectral assay was used.

† The direct-spectral assay (283 nm) was used.

‡ Average value from spectrophotometric and radiochemical assays.

§ These are K_i values from competitive inhibition studies (re IMP) utilizing the coupled-spectral assay, where $K_i = K'_m$ for the alternative-substrate inhibitor [11].

‖ The radiochemical assay was used.

Table 2. Substrate specificity of succino-AMP lyase from *T. cruzi*

Substrate (R_f)*	$K_m \pm$ S.E. (μM)	$V_{max} \pm$ S.E. (relative)	Product (R_f)
Succino-AMP (0.10)	6.0 ± 0.4	100 ± 2	AMP (0.24)
2'-Deoxy-succino-AMP (0.15)	3.0 ± 0.4	75.0 ± 3.5	2'-dAMP (0.29)
Ara-succino-AMP (0.13)	4.8 ± 0.4	35.5 ± 0.9	Ara-AMP (0.24)
8-Aza-succino-AMP (0.10)	<20	20.9 ± 0.9	8-AzaAMP (0.24)
Succino-APPR-MP (0.13)	5.7 ± 0.4	12.8 ± 0.2	APPR-MP (0.27)

* Results from chromatography on cellulose thin-layer plates in 1-propanol–15 M NH_4OH–H_2O, 6:3:1 [6].

substrate specificity that appears to be characteristic of this enzyme from *L. donovani* and many other sources (see Ref. 6 for a review). This enzyme efficiently catalyzed the cleavage of all the succino-AMP analogs. It is interesting that, while the substrates had very similar K_m values, their minimum concentrations required for the protection of succino-AMP lyase against inactivation were very different. This points to probable differences in the parameters that define their K_m and the dissociation constants. This is in agreement with a report that these constants with succino-AMP lyase from yeast are composed of dissimilar combinations of micro-rate constants [12].

In terms of potential chemotherapy, the IMP to AMP pathway of *T. cruzi* is similar to that of *L. donovani* [6] in that it permits the selective amination of HPPR-MP to form APPR-MP. This AMP analog may be the toxic agent or the precursor of the toxic agent that is responsible for the anti-protozoal activity of HPP. 8-Aza-hypoxanthine is a compound with a similar kinetic capacity to be converted into an analog of AMP, but does not display anti-leishmanial activity*.

The results with the ribonucleotide of TPP are of interest. Although this compound was not a substrate for the succino-AMP synthetase of *T. cruzi*, it was capable of inhibiting it with a potency at least equivalent to its potency against the enzyme from *L. donovani* [9]. However, TPP is a considerably weaker growth inhibitor of *T. cruzi**. It is possible that the ability of TPPR-MP to inhibit succino-AMP synthetase may be unrelated to its activity against *Leishmania donovani* even though its intracellular concentration greatly exceeds its K_i value [9]. If the inhibition of succino-AMP synthetase is relevant to its anti-protozoal activity, perhaps *T. cruzi* is inefficient in converting TPP to TPPR-MP. It is also possible that the consequences of such inhibition may be bypassed in *T. cruzi*. Since this organism lacks adenase and adenosine deaminase, it efficiently salvages adenine [13, 14]. On the other hand, the leishmania species are replete in adenase [13, 15, 16] and, therefore, may be more dependent upon succino-AMP synthetase for the conversion of adenine to AMP.

Finally, it is possible that other sites of inhibition exist and may be different in the two genera. Investigations into this possibility are presently in progress.

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* R. L. Berens and J. J. Marr, unpublished data.

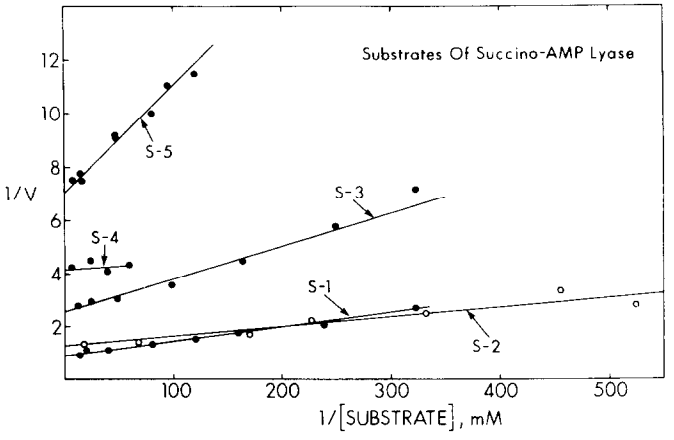

Fig. 2. Double-reciprocal plots of velocity versus substrate concentration. Velocity is expressed as nmoles·min^{-1}·unit^{-1} of succino-AMP lyase. Key: S-1, succino-AMP; S-2, 2'-deoxy-succino-AMP; S-3, ara-succino-AMP; S-4, 8-aza-succino-AMP; and S-5, succino-APPR-MP.

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