# ON A HITHERTO UNKNOWN FERMENTATION PATH OF SEVERAL AMINO ACIDS BY PROTEOLYTIC CLOSTRIDIA

Johann BADER, Peter RAUSCHENBACH and Helmut SIMON
*Institute for Organic Chemistry, Technical University Munich, D-8046 Garching, FRG*

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## 1. Introduction

In the 1930s, Stickland and others [reviews 1,2] described the fermentation of amino acids by numerous species of the genus *Clostridium*. The coupled oxidation and reduction of amino acids constitutes an important energy-yielding process. In this so called Stickland reaction one amino acid (electron donor), e.g. alanine, is oxidized according to the equation:

$$R_2CHCHNH_2COOH + 2\ H_2O \rightarrow$$

$$R_2CHCOOH + CO_2 + NH_3 + 4\ [H] \qquad (1)$$

and the reduction equivalents are taken up by two molecules of an amino acid (electron acceptor):

$$2\ RCH_2CHNH_2COOH + 4\ [H] \rightarrow$$

$$2\ RCH_2CH_2COOH + 2\ NH_3 \qquad (2)$$

Although the metabolic significance was recognized, the enzymes as well as the intermediates involved, especially in the reductive branch of this fermentation, seem only to be known for Gly [3,4] and Pro [5]. The glycine reductase complex synthesizes ATP concomitant with the reductive deamination of Gly [1]. The formation of acyl-phosphates from amino acids as well as from 2-oxo-acylates has been shown by Nisman [6] using several physiological and non-physiological electron acceptors. It is an obvious conclusion that the acyl-phosphates give rise to ATP. However, so far it is not established how amino acids such as Phe, Tyr, Leu take up the four reduction equivalents which are delivered by reaction (1). The formation of the end products expected from the above mentioned amino acids according to reaction (1) and (2) has been described [7–9].

So far neither the stoichiometry with respect to ATP formation nor the rate of the reaction

$$R_2CHCHNH_2COOH + ADP + P_i \rightarrow$$

$$R_2CHCOOH + ATP + CO_2 + NH_3 + 4\ [H] \qquad (1a)$$

or

$$R_2CHCOCOOH + ADP + P_i \rightarrow$$

$$R_2CHCOOH + ATP + CO_2 + 2\ [H] \qquad (3)$$

have been mentioned in the literature.

Herewith we present evidence for a fermentation scheme for a series of amino acids, especially such as Leu, Ile and Phe in which reaction (1) and (2) is coupled to ATP formation and for intermediates as well as products deriving from these amino acids. Furthermore rates of the ATP and end product formation will be given. These are in the same order of magnitude which must be postulated for $Y_{ATP}$ of 10–15 and a doubling time of about 2 h. All the enzyme activities postulated in the scheme could be demonstrated.

## 2. Materials and methods

[U-$^{14}$C] Leu (340 mCi/mmol) and [U-$^{14}$C] Ile (345 mCi/mmol) were from New England Nuclear, Dreieich, (–)D-luciferin, luciferase (both from *Photinus pyralis*) bovine serum albumin, NAD, AMP, ADP, ATP, coenzyme A from Boehringer, Mannheim, *trans*-NRB from Abimed, Düsseldorf, methylviologen

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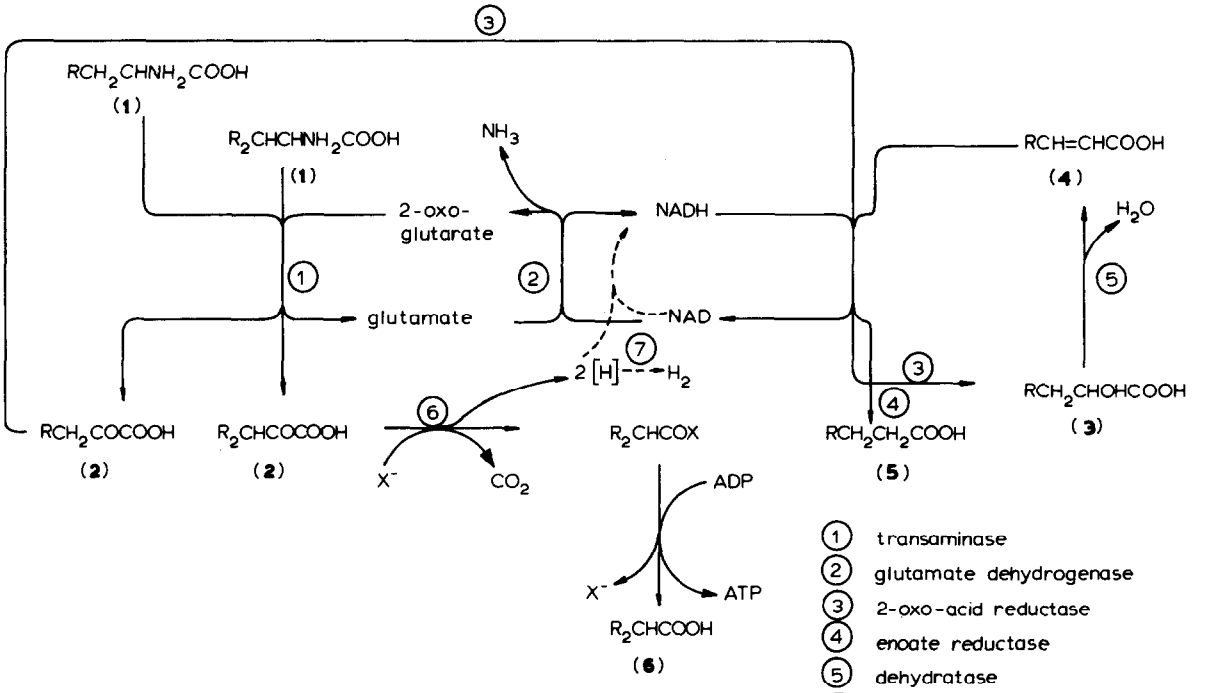
from Ega Chemie, Steinheim, Phe, Ile and Leu and the 2-oxo acids from Degussa, Frankfurt; all other chemicals from Merck, Darmstadt.

*C. sporogenes* ATCC 3584 was grown in a meat broth supplemented with 0.4% peptone (from Casein, Merck), 0.5% yeast extract (Difco), 0.4% Phe, 0.4% Leu or Ile and $4 \times 10^{-5}$ M $FeSO_4$ or in a peptone medium [8,9]. Cultures (150 ml) grown overnight were harvested at 4000 × *g* for 5 min at 30°C in an anaerobic box (Coy, Ann Arbor) and washed once with 30 ml 20 mM potassium phosphate buffer containing 145 mM NaCl and 10 mM $MgSO_4$. Extracts were prepared as in [10]. Fermentations were performed in Warburg vessels. Carbon dioxide was absorbed by 0.4 ml 2 N KOH in the central vessel. All incubations besides the bioluminescence measurements were conducted anaerobically.

Fermentation products were analyzed by HPLC as in [11]. The amounts of the labelled intermediates and end products were determined by measuring the radioactivity of aliquots isolated by HPLC. Phe and its derivatives were spectrometrically measured during their separation by HPLC. The determinations were carefully calibrated. Protein was determined as in [12]. The separation and identification of 2- and 3-methylbutyric acid was conducted on a Carbopack C/0.3%, Carbowax 20M/0.1% $H_3PO_4$ column 200 × 0.2 cm.

ATP measurements were conducted on the basis of recommendations of Boehringer, Mannheim. Samples (10–20 μl) were injected into 0.2 ml stop solution containing 0.1 ml *trans*-NRB and 0.1 ml 0.04 M potassium phosphate buffer (pH 7.5). After mixing 0.1 ml 20 mM Tris–acetate buffer (pH 7.75) containing 0.1 mM EDTA, 5 mM magnesium acetate, 0.5% (w/v) bovine serum albumin, 12 μg (–)D-luciferin, 0.2–0.4 μg protein of luciferase were added. The mixture was immediately counted in a Biolumat LB 9500 (Berthold, Wildbad) for 10 s (integral mode). The values were internally standardized with $5 \times 10^{-11}$ mol of ATP in 50 μl.

Fermentation scheme of amino acids (Phe, Leu, Ile) by Clostridium sporogenes




Sum: $(2-n)\ RCH_2CHNH_2COOH + R_2CHNH_2COOH + ADP + P_i$

$(2-n)\ RCH_2CH_2COOH + R_2CHCOOH + ATP + n\,H_2 + CO_2 + (3-n)\ NH_3$ $(n = 0 \text{ to } 1)$

## 3. Results and discussion

The presented results which lead to the suggested scheme show typical outcomes of many experiments. Due to the lability of the cells and their oxygen sensitivity always freshly grown and harvested cells had to be used. Most of the manipulations including centrifugation were conducted in an anaerobic box in which solutions of reduced methylviologen are stable for many hours. Simultaneous incubation of whole cells with Phe and [$^{14}$C]Ile leads to an accumulation of phenylpropionate and 2-methylbutanoate (table 1). The amount of these products increases from 1–7 h incubation time. Ile is almost exclusively oxidized to 2-methylbutanoate and Phe mostly reduced to 3-phenylpropionate. Incubation of Phe together with [$^{14}$C]Leu reveals again the predominant reduction of the former to phenylpropionate and most of the Leu is oxidized to 3-methylbutanoate. However, depending on the incubation time 13–23% 4-methylpentanoate relative to 3-methylbutanoate can be observed. Besides the acyl-SCoA all the intermediates postulated in the scheme are observed. Taking into account the developed hydrogen the ratio of reduced products:oxidized products is, in the limits of experimental error, not far from unity. Incubation of Leu or Phe without a second amino acid reveals that both amino acids are capable of entering the reductive as well as the oxidative branch of the suggested fermentation. If Ile is incubated without a second amino acid the amount of the oxidation product 2-methylbutanoate is much less as in the presence of Phe. 3-Methylpentanoate could not be detected. The reducing equivalents produced by the formation of 2-methylbutanoate may be taken up by endogenous cell components and by hydrogen formation.

We tried to correlate the ATP formation with the amino acid degradation. In freshly harvested cells suspended in buffer the ATP content decreases rather fast (fig.1). The addition of Phe and Ile leads to a

Table 1

Amounts of substances observed after incubation pairs or single amino acids with whole cells of *C. sporogenes*. Under an atmosphere of nitrogen the Warburg vessels kept at 35° C contained in a total volume of 2.0 ml buffer (section 2) 20 μmol of each of the indicated substrates and cells corresponding to 0.80 mg protein

| Substrate(s) | Time (h) | Intermediates [mM][a] | | | Endproducts [mM][a] | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2-oxo-acid (2) | 2-hydroxy-acid (3) | enoate (4) | reduced carboxylate (5) | oxidized carboxylate (6) | $H_2$[b] | Ratio[c] reduced products / oxidized product |
| [$^{14}$C]Ile + Phe | 1 | <0.01<br>n.d. | 0.15<br><0.01 | <0.01<br>0.002 | <0.01<br>6.1 | 2.9<br>0.02 | 0.9 | 1.2 |
| [$^{14}$C]Ile + Phe | 7 | <0.01<br>0.09 | 0.23<br><0.01 | <0.01<br>0.002 | <0.01<br>9.1 | 5.1<br>0.04 | 3.4 | 1.2 |
| [$^{14}$C]Leu + Phe | 1 | 0.05<br>n.d. | 0.19<br>0.09 | <0.05<br>0.2 | 0.29<br>3.6 | 2.2<br>0.1 | 0.6 | 0.98 |
| [$^{14}$C]Leu + Phe | 7 | n.d.<br>0.02 | 0.62<br>0.04 | 0.16<br>0.02 | 1.65<br>9.6 | 7.2<br>0.04 | 2.3 | 0.95 |
| [$^{14}$C]Ile | 1 | <0.05 | <0.05 | <0.05 | <0.05 | 0.54 | | |
| [$^{14}$C]Leu | 1 | 0.15 | 0.15 | 0.09 | 1.0 | 1.2 | | |
| Phe | 1 | n.d. | 0.05 | 0.37 | 1.9 | 1.1 | | |

[a] Figures given in the same line as the indicated amino acid are products deriving from this amino acid

[b] Amount of $H_2$ is given for 1 ml incubation mixture and therefore comparable with the given concentrations

[c] Formation of (5) and $H_2$ consumes two electrons each that of (6) delivers four electrons per molecule

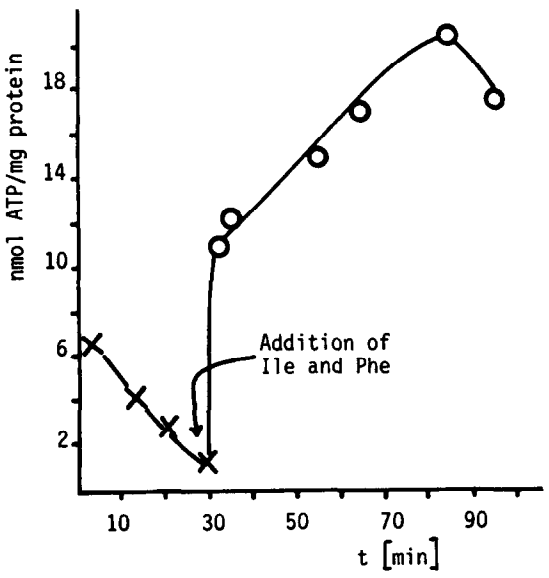



Fig.1. Time course of the ATP content in a cell suspension of *C. sporogenes* in the absence (x – x) and presence of amino acids (o – o). Freshly harvested cells corresponding to 0.12 mg protein were incubated for 30 min at 35°C under an atmosphere of nitrogen in 0.9 ml buffer. After 30 min 0.1 ml buffer containing 10 μmol Ile and 10 μmol Phe were added. The first aliquot was taken after 12 s.

burst of ATP. In 12 s or less the ATP level shoots up from 1 nmol/mg protein to about 11 nmol ATP/mg protein. From there on it increases rather slowly for the next 60 min and decreases afterwards. However, the rate of the formation of the oxidative decarboxylation product **6** stays nearly constant during this time indicating that almost a steady state of synthesis and hydrolysis of ATP is established as soon as the ATP pool reaches its level near to the maximum. Incubation of Ile without a second amino acid does not result in ATP formation.

According to table 2 the rate of ATP formation from Ile or Leu is comparable to that of 2-oxo acids only in the presence of 2-oxo-glutarate. In these experiments methylviologen was used as an artificial electron carrier. It shows about the same redox potential as ferredoxin (–0.42 V) and may replace this in the very dilute solutions. Methylviologen enhances the rates of ATP formation 15–20 fold.

Because of the supplementation with AMP, the formation rates of energy-rich phosphate bonds may be twice as fast as the ATP synthesis rates presented in table 2. *C. sporogenes* shows an approximate doubling time of 2 h. This and an assumed $Y_{ATP}$ value of 10–15 [13] require an ATP formation rate of 1700–1100

Table 2
Some enzyme activities observed in extracts of *C. sporogenes*

| Reaction | Substrates (mM) | Conditions | Activities mU/mg protein |
|---|---|---|---|
| ATP formation | Ile (30) + 2-oxoglutarate (5) | a | 110 |
| ATP formation | Ile (30) without 2-oxoglutarate | a | 2 |
| ATP formation | Leu (30) + 2-oxoglutarate (5) | a | 150 |
| ATP formation | (35) -3-methyl-2-oxopentanoate (20) | a | 150 |
| ATP formation | 4 -methyl-2-oxopentanoate (20) | a | 150 |
| ATP formation | Phenylpyruvate (20) | a | 15 |
| Transaminase | One of the amino acid Ile, Val, Leu, Phe (20) or Tyr (5) + 2-oxoglutarate (10) | b | 320–490 |
| Transaminase | Ile without 2-oxoglutarate | b | 10 |
| Glutamate dehydrogenase | Glu (2.3) + NAD (1.5) | c | 2700 |

All reactions were carried out under anaerobic conditions at 35°C
(a) 1.0 ml phosphate buffer, 75 mM (pH 7.5), contained: 10 mM NAD, 15 mM methyl-viologen, 0.5 mM coenzyme A, 0.65 mM thiamine pyrophosphate, 0.3 mM pyridoxal phosphate, 10 mM $MgSO_4$, 0.135 mM AMP, 0.01 mM ADP and 1.4 mg protein of the 40 000 × *g* supernatant. (Omission of coenzyme A or phosphate or $Mg^{2+}$ reduced the ATP formation to 2–10%)
(b) 2.0 ml phosphate buffer, 40 mM (pH 7.3), contained 0.033 mM pyridoxal phosphate and 1.9 mg protein. The glutamate was determined according to [18]
(c) 2.0 ml phosphate buffer, 75 mM (pH 8.0), contained 0.2 mM pyridoxal phosphate and 0.10 mg protein after removing low molecular components by ultrafiltration

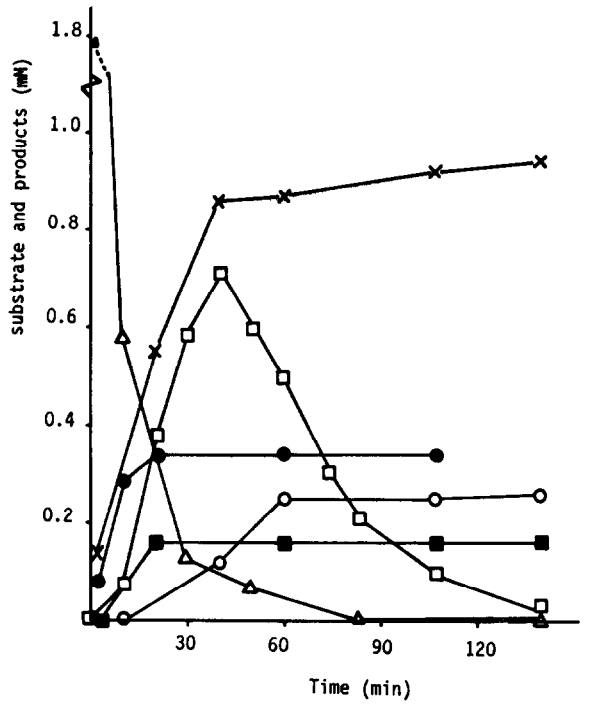



Fig.2. Formation of ATP (□□) during the disproportionation of phenylpyruvate (△△). A 40 000 × *g* supernatant of a crude extract of *C. sporogenes* (1.4 mg protein/ml) was incubated with 2 mM phenylpyruvate and 1 mM AMP. Phenylacetate (xx), phenyllactate (●●), phenylpropionate (○○), cinnamate (■■).

nmol . $min^{-1}$ . mg $protein^{-1}$. Taking the highest value from table 2 (300 nmol energy-rich bonds . $min^{-1}$ . mg $protein^{-1}$) about 20–30% of the growth rate could be explained. Due to the complexity of the growth medium [2] which is necessary for growth an exact determination of $Y_{ATP}$ is hard to achieve.

After addition of Phe and Ile whole cells fill up their ATP pool in 12 s or less (fig.1). This means a rate of ATP formation of ⩾60 nmol ATP . $min^{-1}$ . mg $protein^{-1}$. Fig.2 reveals evidence for a stoichiometric relationship between phenylpyruvate consumption, ATP and phenylacetate formation as well as for the consumption of reducing equivalents in the form of phenylpropionate and phenyllactate formation. Therefore one can assume that in whole cells the ATP formation can be as fast as the formation of the other products according to the scheme. Since *C. sporogenes* demands a complex medium, several amino acids may be degraded simultaneously to produce ATP. Using a rapid sample device Kröger and Winkler [14] measured initial rates of ATP formation in *Vibrio succinogenes* of 3 nmol . mg $protein^{-1}$ . 5 $s^{-1}$ at 15°C during the reduction of fumarate at the cost of the AMP level. This value is rather similar to that which we found for *C. sporogenes.*

The observed transaminase activities (table 2), that for Glu dehydrogenase together with the fact that dehydrogenase activities for Ile, Leu, Val and Phe could hardly be detected (2–14 mU/mg protein) indicate that the 2-oxo-acids are formed via transamination as depicted in the scheme.

*C. sporogenes* possesses all the enzyme activities for the reductive branch [15]. The different behaviour of amino acids with respect to oxidation or reduction seems to be determined by the substrate specificity of the enoate reductase and the 2-oxo-acid reductase in *C. sporogenes*. (*E*)- as well as (*Z*)-3-methylpentenoate are no substrates for the enoate reductase whereas cinnamate or 4-methyl-2-pentenoate are reduced [15]. The specific activity of a crude extract for the reduction of cinnamate is about 200–300 mU/mg protein [15]. This value is comparable with the rates indicated in table 2 and those which can be calculated from table 1 taking into account the products after 1 h. The activity of the NAD(P) dependent 2-oxo acid reductase in crude extract is about one order of magnitude higher for phenylpyruvate or 4-methyl-2-oxopentanoate. The oxo analogue of Ile is reduced at a much lower rate [16]. Though the water elimination from (2*R*)-2-hydroxy acids is mechanistically still unclear, there is no doubt that this reaction occurs [15]. The formation of glutaconate from (2*R*)-2-hydroxyglutarate by extracts of *A. fermentans* as proved in [17] is an analogous reaction. We have preliminary evidence that Val behaves like Ile and Tyr in the same way as Phe.

Our findings can be reasonably explained by the presented scheme and are in agreement with observations in [7–9]. The enoate reductase plays an important role in the reductive branch of the scheme. In the meantime we detected enoate reductase in other proteolytic clostridia and according to reports in [8,9] and our own observations the end products (**5**) and (**6**) of table 1 are formed. Therefore we assume that the suggested fermentation scheme plays a role in many proteolytic clostridia.

If one tries to write an equation for the scheme one realizes immediately that there may be a difficulty since there is no stoichiometric hydrogen evolution (table 1). There is a branching point at the conversion

of (2) to (6). Reducing equivalents may be taken up by protons or finally by pyridine nucleotide dependent reductions. That means the amount of amino acids used per molecule of formed ATP depends on the amount of developed hydrogen.

This dependence can be expressed in the equation given in the scheme. In the experiments summarized in table 1, $n$ was ~0.3 for the first hour.

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## References

[1] Seto, B. (1980) Diversity Bact. Respir. Syst. 2, 49–64.
[2] Barker, H. A. (1981) Annu. Rev. Biochem. 50, 23–40.
[3] Turner, D. C. and Stadtman, T. C. (1973) Arch. Biochem. Biophys. 154, 366–381.
[4] Tanaka, H. and Stadtman, T. C. (1979) J. Biol. Chem. 254, 447–452.
[5] Seto, B. and Stadtman, T. C. (1976) J. Biol. Chem. 251, 2435–2439.
[6] Nisman, B. (1954) Bacteriol. Rev. 18, 16–42.
[7] Moss, C. W., Lambert, M. A. and Goldsmith, D. J. (1970) Appl. Microbiol. 19, 375–378.
[8] Elsden, S. R., Hilton, M. G. and Waller, J. M. (1976) Arch. Microbiol. 107, 283–288.
[9] Elsden, S. R. and Hilton, M. G. (1978) Arch. Microbiol. 117, 165–172.
[10] Bader, J. and Simon, H. (1980) Arch. Microbiol. 127, 279–287.
[11] Giesel, H., Machacek, G., Bayerl, J. and Simon, H. (1981) FEBS Lett. 123, 107–110.
[12] Herbert, D., Phipps, P. J. and Strange, R. E. (1971) in: Methods in Microbiology, vol. 5B (Norris, J. R. and Ribbons, D. W. eds) pp. 209–344, Academic Press, London, New York.
[13] Thauer, R. K., Jungermann, K. and Decker, K. (1977) Bacteriol. Rev. 41, 100–180.
[14] Kröger, A. and Winkler, E. (1981) Arch. Microbiol. 129, 100–104.
[15] Bühler, M., Giesel, H., Tischer, W. and Simon, H. (1980) FEBS Lett. 109, 244–246.
[16] Machacek, G., Bader, J. and Simon, H. (1982) in preparation.
[17] Buckel, W. (1980) Eur. J. Biochem. 106, 439–447.
[18] Bernt, E. and Bergmeyer, H. U. (1974) in: Methoden der enzymatischen Analyse (Bergmeyer, H. U. ed) vol. II, pp. 1749–1759, Verlag Chemie, Weinheim.