Flavine Mononucleotide

Herbert C. Friedmann

Principle

Lactic oxidase from pneumococci catalyses the reaction:

(1) $$\text{Lactate} + O_2 \longrightarrow \text{acetate} + CO_2 + H_2O$$

The cofactor for the enzyme is flavine mononucleotide (FMN). The FMN content of the sample is determined by the activation of the apoenzyme [1]. The reaction is followed in a Warburg manometer, the oxygen uptake per unit time being an indication of the rate of the reaction. By plotting the reaction rates with standards against the FMN concentration according to *Michaelis-Menten* or *Lineweaver-Burk* [1a] a standard curve is obtained. The method is similar to the determination of flavine adenine dinucleotide (FAD) with the apoenzyme of D-amino acid oxidase [2,3]. The Michaelis constant of the lactic oxidase apoenzyme for FMN is 4.8×10^{-7} M [1].

Reagents

1. Sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2\ H_2O$
3. Potassium hydroxide, 5 N
4. Lithium-DL-lactate
5. Flavine mononucleotide
   sodium salt, FMN-NaH $\cdot 2\ H_2O$; commercial preparation, see p. 1013.
5. Lactic oxidase apoenzyme
   according to [1] from *Diplococcus pneumoniae* R 36A. For an outline of the method of preparation, see Appendix, p. 601.

Preparation of Solutions

I. Phosphate buffer (1.0 M; pH 7.1):
Dissolve 138.0 g. $NaH_2PO_4 \cdot H_2O$ and 178.05 g. $Na_2HPO_4 \cdot 2\ H_2O$ in distilled water and make up to 1000 ml.

II. DL-Lactate (1.0 M):
Dissolve 0.9601 g. lithium-DL-lactate in distilled water and make up to 10 ml.

III. Flavine mononucleotide, FMN
   a) Stock solution (3.5×10^{-4} M):
   Dissolve 9 mg. FMN-NaH $\cdot 2\ H_2O$ in distilled water and make up to 5 ml. Check the concentration spectrophotometrically (extinction coefficient of FMN [4] is 11.3 cm.^{2}/mmole at 450 mμ and pH 7).
   b) Standard solution (7×10^{-6} M):
   Immediately before use dilute 0.1 ml. solution a) to 50 ml. with distilled water.

---

[1] *S. Udaka, J. Koukol* and *B. Vennesland*, J. Bacteriol. *78*, 714 [1959].
[1a] *H. Lineweaver* and *D. Burk*, J. Amer. chem. Soc. *56*, 658 [1934].
[2] *O. Warburg* and *W. Christian*, Biochem. Z. *298*, 150 [1938].
[3] *F. B. Straub*, Biochem. J. *33*, 787 [1939].
[4] *F. M. Huennekens* and *S. P. Felton* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 950.

IV. Apoenzyme:
Preparation of the solution, see Appendix, p. 601.

Stability of the solutions

Store the FMN stock solution in a brown bottle. The solutions of the apoenzyme, lactate and FMN keep for several months at −15°C.

Procedure

The conditions for the extraction of FMN are the same as those for FAD (see p. 597).

Manometric measurements

Warburg manometers; vessels with centre wells and side arms; gas phase: air; temperature: 30°C. The following vessels are required for each determination: 2—3 experimental vessels, 3—4 standard vessels, 1 control vessel (FMN-free) and 1 thermobarometer.

Set up the vessels as follows:

		Experimental and Standards	Control	Thermo-barometer
Main compartment	buffer (soln. I)	0.1 ml.	0.1 ml.	—
	apoenzyme (soln. IV)	0.1 ml.	0.1 ml.	—
	sample or standard soln. (IIIb)	1.7 ml.	—	—
	distilled water	—	1.7 ml.	2.1 ml.
Side arm	lactate (soln. II)	0.1 ml.	0.1 ml.	—
Centre well	5 N KOH (on filter paper)	0.1 ml.	0.1 ml.	—

Allow the vessels to stand for 30 min. in the dark at room temperature (re-activation of the apoenzyme by the FMN). Then equilibrate the vessels for 10 min. at 30°C. Tip the contents of the side arm into the main compartment and close the manometer tap. Start a stop-watch and read the manometer levels (h) every 5 or 10 min. Calculate the Δh/min. and average the values.

Calculations

The oxygen uptake $\Delta 0_2$/min. for the experimental and standard vessels is calculated from the manometer readings (mm. manometer fluid) by multiplying by the vessel constants k (after correction for changes in the thermobarometer and control).

Plot the $\frac{1}{\Delta 0_2/\text{min.}}$ for the standards against the $\frac{1}{\text{FMN content}}$. Obtain the FMN content of the experimental vessels from this standard curve.

Other Methods for the Enzymatic Determination of FMN

FMN can also be determined spectrophotometrically by means of its activation of the apoenzyme of the TPNH cytochrome c reductase[5] (modification:[4]).

Appendix

Preparation of lactic oxidase[1]

Add solid ammonium sulphate up to 50% saturation to an autolysate from *Diplococcus pneumoniae* R 36 A at 0 to 4°C. Centrifuge and discard the precipitate. To the supernatant add solid ammonium sulphate to give 68% saturation. Centrifuge and dissolve the precipitate in a solution of 0.02 M Na_2HPO_4 containing 10^{-3} M EDTA. Dialyse for 3 hours with stirring against this solution.

[5] *E. Haas, B. L. Horecker* and *T. R. Hogness*, J. biol. Chemistry *136*, 747 [1940].

Preparation of the apoenzyme[1)]

To 10 ml. of a solution of lactic oxidase containing 2800 units*) (specific activity: 35 units/mg.) add 3.5 ml. saturated ammonium sulphate solution. Very slowly add 4.5 ml. 0.1 N H_2SO_4 to this mixture in the cold, allow to stand for 15 min. at 0° C and then centrifuge. Wash the precipitate with 5 ml. saturated ammonium sulphate solution and dissolve it in 10 ml. 0.1 M phosphate buffer (pH 7.2).

---

*) A unit[1)] is the amount of enzyme which causes an oxygen uptake of 1 μmole/hour at 30° C in a reaction mixture containing 100 μmoles phosphate buffer (pH 7.2) and 100 μmoles Li-DL-lactate in a final volume of 2.8 ml.