The Preparation and Properties of an Enzyme Electrode for Creatine

Junko MOTONAKA,* Hisaaki TAKABAYASHI, Sanae IKEDA, and Nobuyuki TANAKA†
Technical College, The University of Tokushima, Minamijosanjima, Tokushima 770
†Nippon Jimuki Co., Ltd., 12, 3-Chome, Kanda Ogawa-cho, Chiyoda-ku, Tokyo 101
(Received December 17, 1987)

Synopsis. An enzyme electrode for creatine consisting of a single glass electrode and immobilized creatine kinase was prepared, and the effects of various conditions on the calibration curve obtained with this electrode were examined. Creatine in the range of 0.42—3.3 mmol dm^{-3} could be determined at 37 °C and pH 9.

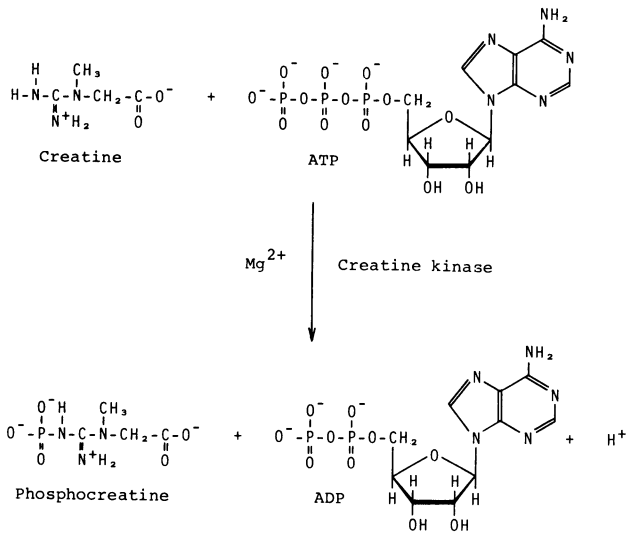
Creatine is a nitrogenous compound produced by protein metabolism. It is found in muscle in combination with phosphoric acid as phosphocreatine and is intimately associated with the chemical changes which take place during muscle contraction. Creatine is not found in any appreciable amount in the urine. There is a little in the urine of women and children, but not normally in that of men.[1] It is increased by the destruction and denaturation of muscles. The creatine content in urine or serum can thus be used clinically.

There has been much recent progress in the study of enzyme electrodes,[2-8] but an enzyme electrode for creatine has not yet been developed. In this work, we prepared an enzyme electrode for the determination of creatine. The enzyme reaction is shown in Scheme 1.

Experimental

Apparatus and Reagents. A glass electrode, Model GST-155C (TOA Electronics, Ltd.), was employed to prepare the enzyme electrode. The pH was measured with a digital pH/mV meter, Model TSC-10A (TOA Electronics, Ltd.), connected with an electronic recorder, Model R-102 (Rikadenki Kogyo Co., Ltd.). All the measurements were carried out at 37±0.2 °C.

The creatine kinase (from rabbit muscle; specific activity, 800 units per mg at 38 °C) was obtained from Boehringer Mannheim Yamanouchi, and the adenosine 5′-triphosphate (ATP)(Purity is 98±3%), from the Oriental Yeast Co. The creatine (analytical-reagent grade), bovine serum albumin (BSA)(Fraction V powder; purity, 98.5±0.5%), and glutaraldehyde(25%) were obtained from Wako Pure Chemical Industries. The ATP(10^{-1} mol dm^{-3}), creatine (3×10^{-2} mol dm^{-3}), BSA(10 w/w%), and glutaraldehyde (12.5 v/v%) solutions were prepared with distilled water.



Scheme 1. Enzyme reaction of creatine.

Preparation of the Enzyme Electrode. The electrode consisted of a glass electrode with an immobilized creatine kinase membrane fitted to its detector end. A cross-linking method was used for the immobilization of the creatine kinase. That is, 30 µl of a 10 w/w% BSA solution was spread on a polycarbonate membrane (0.1 µm caliber), after which 5 mg of creatine kinase and 4 µl of 12.5 v/v% glutaraldehyde were added with prompt mixing. They were spread at a thickness of 0.05 mm. The membrane was then left to stand for at least 2 hours at 4 °C and then attached to the glass electrode by means of an O-ring.

Determination of Creatine. The enzyme electrode for creatine was immersed in 10 ml of a 2 mmol dm^{-3} ATP-0.1 mmol dm^{-3} magnesium sulfate (pH 9) solution in a thermostat-regulated cell. Then 2 ml of the creatine solution was injected into the cell, and the ion-production by the enzyme reaction during the first 10 minutes, unless otherwise indicated, was monitored as the change in the pH. The electrode was washed with distilled water in each measurement.

Results and Discussion

Immobilization of Enzyme. A cross-linking method and matrix-entrapping methods of enzyme immobilization were also examined. (A) Cross-linking method: The membrane was prepared as has been described in the experimental sections. (B) Matrix-entrapping method: On a glass plate, 10 µl of a 20 w/w% cellulose acetate–acetone solution and 30 µl of a creatine kinase aqueous solution (5 mg/30 µl) were mixed quickly and spread at a thickness of 0.05 mm. This immobilized enzyme membrane and a polycarbonate membrane with a caliber of 0.1 µm were attached to a single glass electrode by means of an O-ring.

The response curves of the two types of electrodes are shown in Fig. 1. The response curves of Electrodes A and B were similar. The ΔpH value (0.18) of Electrode A after 10 min was greater than that (0.11) of Electrode B. The caribration curve of Electrode A was also compared with that of Electrode B [Fig. 2, Curves (A) and (D)]. Both methods (cross-linking and matrix-entrapping) could be employed. Further studies were made on the cross-linking method with greater ΔpH values.

Effects of Alkaline Solutions. In the enzyme reaction shown in Scheme 1, the optimum pH of creatine kinase is pH 8—9.[9] The sample solution was adjusted to this pH with sodium hydroxide, aqueous

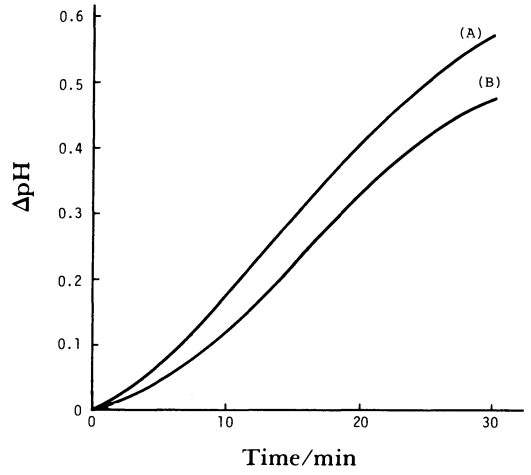


Fig. 1. Response curves of creatine enzyme electrode. (A) Cross-linking method. (B) Matrix-entrapping method. The sample (12 ml) contained 1.67 mmol dm^{-3} creatine, 1.67 mmol dm^{-3} ATP, and 8.33 mmol dm^{-3} magnesium sulfate. Temp 37 °C.

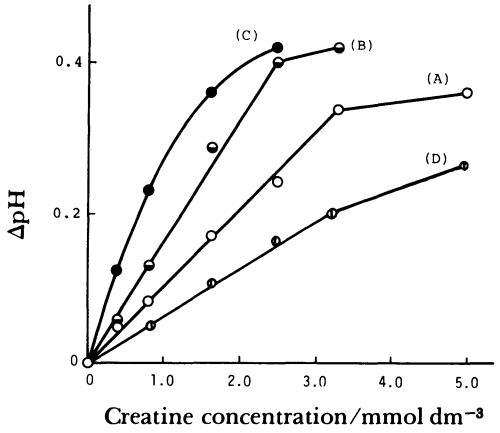


Fig. 2. Calibration curves of creatine. (A) Cross-linking method (after 10 min). (B) Cross-linking method (after 20 min). (C) Cross-linking method (after 30 min). (D) Matrix-entrapping method (after 10 min). The sample (12 ml) contained the indicated amount creatine, 1.67 mmol dm^{-3} ATP and 8.33 mmol dm^{-3} magnesium sulfate. Temp 37 °C.

ammonia, or potassium hydroxide. The response (ΔpH) was greatest with potassium hydroxide (0.18) and least with sodium hydroxide (0.03), because sodium hydroxide inactivated creatine kinase.[10] Potassium hydroxide was used in the subsequent experiments.

Effects of ATP and Magnesium Sulfate Concentrations. ATP plays an important part in energy metabolism, and the magnesium ion functions as a cofactor of creatine kinase.[11] The responses were measured with concentrations of 8.3×10^{-3}—8.33 mmol dm^{-3} ATP and 0.833—41.7 mmol dm^{-3} magnesium sulfate for 1.67 mmol dm^{-3} of creatine. The ΔpH values increased with the ATP concentration in the range of 8.33×10^{-3}—1.67 mmol dm^{-3} and was constant in the range of 1.67—8.33 mmol dm^{-3} (the amount required theoretically was 1.67 mmol dm^{-3} ATP), because at higher concentrations the amount of ATP passing through the membrane became constant. Similarly, the ΔpH increased with up to 8.33 mmol dm^{-3} magnesium sulfate, and then became constant because the magnesium-ion concentration was in excess of the amount required as a cofactor for the creatine kinase. Accordingly, 1.67 mmol dm^{-3} ATP and 8.33 mmol dm^{-3} magnesium sulfate were used.

Effect of the Temperature. The effect of the temperature on the ΔpH was also examined in the range of 20—40 °C. The ΔpH value (0.180 after 10 min) was the greatest at 37—38 °C. The creatine kinase was inactivated at 40 °C (ΔpH was 0.120). The optimum temperature for creatine kinase was 37—38 °C. Therefore, all subsequent measurements were done at 37 °C.

Effect of the Amount of Immobilized Creatine Kinase. The effects of the amount of immobilized enzyme will also be discussed. Creatine kinase in the range from 2000 units (2.5 mg) to 8000 units (10 mg) was immobilized with 6 μl of a 10 w/w% BSA solution and 0.8 μl of a 12.5 v/v% glutaraldehyde solution per 1 mg of enzyme. The value of ΔpH increased in the range of 2000—4000 units of the enzyme and remained constant with larger amounts. The enzyme activity was low with small amounts of enzyme because a part of the active site reacted with the cross-linking agent.[12] On the basis of these results, 5 mg (4000 units) of creatine kinase were immobilized with 30 μl of a 10 w/w% BSA solution and 4 μl of a 12.5 v/v% glutaraldehyde solution.

Calibration Curve. Linear plots of the creatine concentration vs. the ΔpH were obtained at concentrations of 0.42—3.3 mmol dm^{-3} at 10 min [Curve (A)], 0.42—2.5 mmol dm^{-3} at 20 min [Curve B)] and 0.42—1.6 mmol dm^{-3} at 30 min [Curve (C)], as is shown in Fig. 2. The plots fit these three equations; $Y=96.66X+0.006$ [Curve (A)], $Y=165.8X-0.006$ [Curve (B)], and $Y=183.3X-0.06$ [Curve (C)], where Y is ΔpH and X is the creatine concentration in mmol dm^{-3}. The correlation coefficients were 0.999, 0.998, and 0.991 respectively. The coefficients of variation were less than 7.2%.

Life-Time of the Electrode. The life-time of the prepared enzyme electrode for creatine was also considered. The stability of this electrode is good for 0—3 days (ΔpH was 0.18) after the preparation. However, the sensitivity of this electrode decreased after 4 days (the ΔpH values were 0.169, 0.150, 0.135, 0.117, 0.103, and 0.086 after 4, 5, 6, 7, 8, and 9 days respectively) because of the decrease in the activity of the enzyme. Just this electrode was used 10—20 times a day.

Effects of Concomitant Compounds. The creatine was determined in the presence of eight other compounds (creatinine, β-D-glucose, cholesterol, sodium chloride, urea, L-ascorbic acid, iodine, and citric acid), as is shown in Table 1. Creatinine, glucose, cholesterol, and sodium chloride were present in mole amounts about 10, 100, and 1000 times respectively

Table 1. Effects of Concomitant Compound on the Determination of Creatine

Concomitant compound	Molar ratio creatine : concomitant compound (mol : mol)	Relative error (%)
Creatinine	1 : 1	−1.0
	1 : 10	0.4
	1 : 100	0.9
β-D-Glucose	1 : 1	0.5
	1 : 10	0.3
	1 : 100	0.5
Cholesterol	1 : 0.01	−0.2
	1 : 0.05	−0.4
Sodium chloride	1 : 1	0.7
	1 : 100	−0.5
	1 : 1000	0.9
L-Ascorbic acid	1 : 1	0.6
	1 : 3	1.1
	1 : 5	−1.3
Iodine	1 : 0.1	−3.0
	1 : 0.5	−2.4
Citric acid	1 : 0.1	−2.2
	1 : 0.5	−3.0
Urea	1 : 1	−1.0
	1 : 10	−1.7
	1 : 100	2.9

Sample, 12 ml, of 1.67 mmol dm^{-3} creatine 1.67 mmol dm^{-3} ATP and 8.33 mmol dm^{-3} magnesium sulfate. Temp 37 °C.

that of creatine in human serum. Creatine could be determined with a relative error of less than ±1.7% in the presence of molar ratios of 1—100 creatinine, 1—100 β-D-glucose, 0.01—0.05 cholesterol, 1—1000 sodium chloride, 1—5 L-ascorbic acid, and 1—10 urea. The effect of cholesterol was examined at less than the presence ratio in human serum, because the solubility of cholesterol was small (0.2 mg/100 ml H_2O). Creatine could be determined with a relative error of less than −3% in the presence of molar ratios of 0.1—0.5 iodine as the SH reagent and 0.1—0.5 citric acid as the chelating agent.

References

1) I. Kanai, "Rinsho Kensaho Teiyo," ed by M. Kanai, Kodansha, Tokyo (1983), p. 428.
2) "Ion Denkyoku to Koso Denkyoku," ed by S. Suzuki, Kodansha, Tokyo (1981), pp. 65, 187.
3) M. Koyama and Y. Sato, *Anal. Chim. Acta*, **116**, 307 (1980).
4) D. P. Nikolelis and T. P. Hadjiioannou, *Anal. Chim. Acta*. **147**, 33 (1983).
5) G. G. Guilbault, S. P. Chena, and S. S. Kuan, *Anal. Lett.*, **13**, 1607 (1980).
6) G. G. Guilbault, B. Danielsson, C. F. Mandenius, and K. Mosbach, *Anal. Chem.*, **55**, 1582 (1983).
7) M. Meyerhoff and G. A. Rechnitz, *Anal. Chim. Acta*, **85**, 277 (1976).
8) G. J. Papariello, A. K. Mukherji, and C. M. Shearer, *Anal. Chem.*, **45**, 790 (1973).
9) "Koso Handbook," ed by Y. Kajiro, M. Tokushige, T. Yagi, and E. Ichishima, Asakura Shoten, Tokyo (1983), p. 372.
10) T. A. Mahowalb, E. A. Noltmann, and S. A. Koby, *J. Biol. Chem.*, **237**, 1535 (1962).
11) "Rinsyo Koso Handbook," ed by S. Baba, H. Wada, M. Kitamura, and J. Okuda, Kodansha, Tokyo (1982), p. 313.
12) "Ion Denkyoku to Koso Denkyoku," ed by S. Suzuki, Kodansha, Tokyo (1981), p. 67.