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Sarcosine oxidase: structure, function, and the application to creatinine determination

Review Article

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Summary. Determination of creatinine is important in the clinical laboratory. Jaffé reaction has long been used to determine creatinine, but the method suffers from various interferences. To overcome this problem, the enzymatic methods were invented and have been used widely. Sarcosine oxidase has a critical role in the enzymatic method. Of sarcosine oxidases, *Corynebacterium* enzyme has been studied extensively in kinetic and structural aspects. The enzyme contains noncovalently bound and covalently bound FADs, and consists of 4 non-identical subunits (A, B, C, D). The covalently bound FAD is bound to the subunit B. The rate of oxidation of sarcosine was explained by the rates of the oxidation and reduction of the bound FADs. From the chemical modification of the enzyme with iodoacetamide, the amino acid sequence around the non-covalently bound FAD is suggested and the modification changed the enzyme so that the only noncovalently bound FAD functions in the oxidation of sarcosine.

Keywords: Amino acids – Creatine – Creatinine – Chemical and enzymatic determination – Sarcosine oxidase – Covalent and noncovalent FAD – Reaction mechanism

1. Introduction

Phosphocreatine, a high energy phosphate compound, plays a unique role in the energetics of muscle and other excitable tissues, such as brain and nerve. This compound serves as a reservoir of high energy phosphate groups. Phosphocreatine has a higher energy group than ATP, then this compound can transfer its phosphate group to ATP in a reaction catalyzed by creatine kinase. During the period of rest, this enzyme catalyzes to form phosphocreatine. Creatine is synthesized in liver and kidney from Arg, Gly, and Met as shown in Fig. 1. The

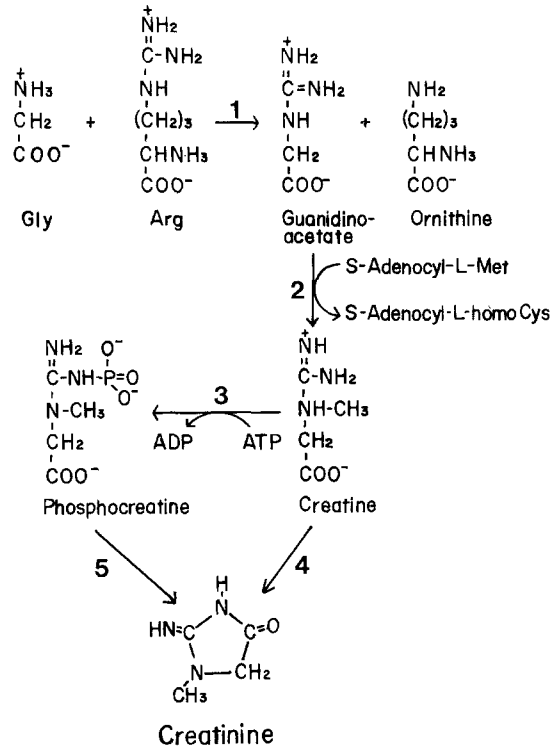


Fig. 1. Metabolism of creatine and creatinine. **1** EC 2.1.4.1 L-Arginine: Glycine amidinotransferase (kidney). **2** EC 2.1.1.2 S-adenosylmethionine : guanidinoacetate N-methyl transferase (liver). **3** ATP: creatine phosphotransferase (muscle). **4, 5** nonenzymatic

transfer of amidine group from Arg to Gly produce guadininoacetate and ornitine. The reaction is catalyzed by transamidinase, which can be prepared from kidney. Creatine is formed by the addition of a methyl group to guanidinoacetate from S-adenosylmethionine. A methyltransferase is required in this reaction. Most of creatine synthesized is transported to muscle through vein and phosphorylated to form phosphocreatine. Creatine and phosphocreatine are converted to their anhydride, creatinine nonenzymatically. Creatinine diffuses from muscle and is excreted almost renally in the urine. The serum creatinine concentration varies inversely with the glomerular filtration rate. Therefore, serum creatinine concentration is widely used as an indicator of renal function. On the other hand, much of creatine is reabsorbed renally and it is not secreted in urine under normal conditions.

Determination of creatinine and creatine was therefore important in the clinical laboratory. The chemical method, Jaffé reaction, has been widely used for the determination of creatinine and creatine, but the method suffers from various interferences. Recently, the enzymatic methods have been used because of its simplicity and a few interference substances. Sarcosine oxidase has a critical role in one of the most reliable enzymatic methods. In our laboratory, this enzyme has been studied extensively on the kinetic and structural aspects.

In this review, I summarize the methods of creatinine determination and describe the studies on the kinetic and structural aspects of sarcosine oxidases.

2. Creatinine assay

2.1 Creatine and creatinine levels in disease

Creatine is an important constituent in muscle, brain, and blood in the form of phosphocreatine (Walker, 1979). Normally, small amounts of creatine are excreted in the urine, but in the states of elevated catabolism and in the presence of muscular diseases, the excretion increases. Erythrocyte creatine level has been correlated with erythrocyte age (Grifiths et al., 1967; Fehr and Knob, 1986; Buysse et al., 1990) and it is of potential value in the diagnostic evaluation of hemolytic anemia (Cramer et al., 1987).

Creatinine in serum is elevated in renal insufficiency, urinary tract obstruction and impairment of renal function (for review see: Narayanan and Appleton, 1980; Bowers and Wong, 1980; Spencer, 1986). Because urine is derived from kidney, the glomerular filtration rate is extremely important in the assessment of renal function. Creatinine clearance (=[Urinary Creatinine][Urine Flow]/[Plasma Creatinine]) calculated from measured creatinine concentrations in serum and urine indicates renal dysfunction more sensitively. Normal value of the clearance is 90–120 ml/min in healthy adults (Murray, 1987).

2.2 Creatinine determination

Since creatine concentration can be determined by the modified method of creatinine determination, mainly creatinine determination is considered in this review.

2.2.1 Chemical method

Jaffé (1886) first described the red color reaction when creatinine reacted with picrate in an alkaline medium (Fig. 2). Folin (1904) used Jaffé reaction to measure creatinine in urine and deproteinized blood. Since then, Jaffé reaction became the common method to measure serum and urinary creatinine. However, even now the chemistry of this reaction is not clear, and the structure of the product and the mechanism are still a matter for debate (Butler, 1977; Vasiliades, 1977; for review see Narayanan and Appleton, 1980; Spencer, 1986). Figure 2 shows the structures of the reaction products proposed. One is a 1:1 Janovsky type complex ((1) in Fig. 2) of picrate and creatinine, which is then converted to a stable yellow bis complex (Butler, 1975; Vasiliades, 1976). The second is a 1:1 creatinine/picrate adduct ((2) in Fig. 2; Seelig, 1969; Vasiliades, 1976). Against these proposals, Kroll et al. (1987) presented an interesting mechanism of Jaffé reaction. They studied the spectral, kinetic and equilibrium properties on the reaction of the picrate with creatinine and with cyclic and aliphatic ketones, and found that the common structure for all of the compounds reacting with picrate is the carbonyl group. They proposed that a co-planar charge transfer complex is formed between picrate and creatinine (or ketones) in the Jaffé reaction ((3) in Fig. 2).

The reaction of picric acid and creatinine is known to be nonspecific and the method suffers from various interferences (Narayanan and Appleton, 1980; Bowers and Wong, 1980; Spencer, 1986; Murray, 1987; Bruns, 1988; Watts and

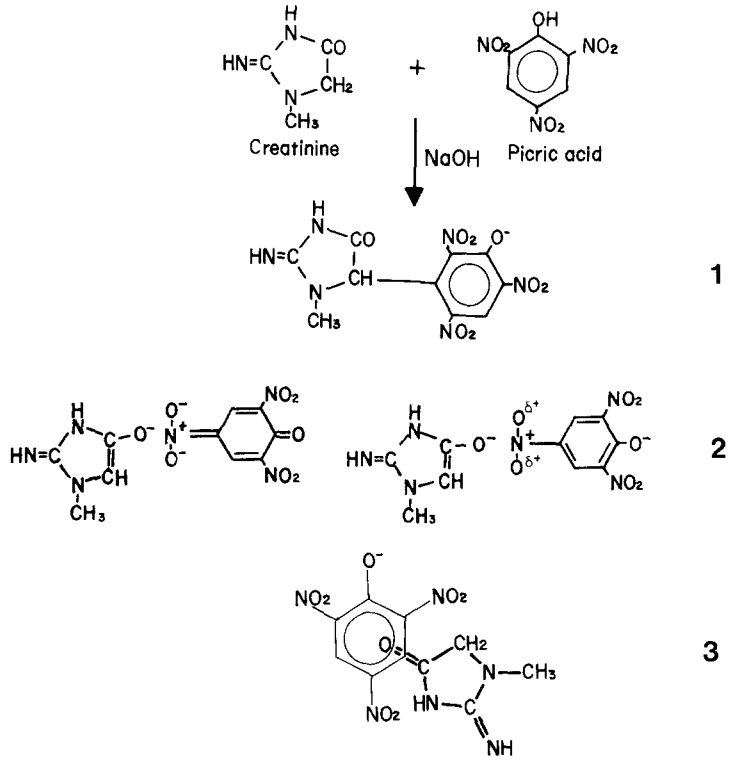


Fig. 2. Jaffé reaction. **1** Janovski type complex (Butler, 1975). **2** A 1:1 creatinine/picrate adduct (Seelig, 1969; Vasiliades, 1976). **3** A co-planar charge transfer complex (Kroll et al., 1987)

Pillay, 1990; Jacobs et al., 1991). The potential interferences are acetoacetic acid, acetone, bilirubin, cephalosporin antibiotics, glucose, hemoglobin, and lipids. To overcome this problem, the enzymatic methods have been invented for the creatinine determination.

2.2.2 Enzymatic methods

The enzymatic method was first reported by Miller and Dubos (1937). They were able to measure the creatinine content of plasma and urine with a crude mixture of creatininase (creatinine amidohydrolase, EC 3.5.2.10) and creatinase (creatine amidino hydrolase, EC 3.5.3.3). The difference in color produced by Jaffé reaction in the enzyme-treated and untreated samples was attributable to the true creatinine content in the sample. Since then, various enzymatic methods were reported (Spencer, 1986). These can be classified into three groups depending upon which enzyme or combination of enzyme were used.

2.2.2.1 Methods using creatininase

Wahlefeld et al. (1972) first reported a complete enzymatic method to measure creatinine in plasma and urine. Using a following multienzyme system, they measured creatinine by monitoring the absorbance at 340 nm.

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatininase}} \text{Creatine}$$

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{Creatine kinase}} \text{Phosphocreatine} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + NAD^+$$

The rate of change in absorbance at 340 nm due to oxidation of NADH is directly proportional to the amount of creatinine present. A number of authors have applied this method for the measurement of creatinine in the plasma and urine and showed that it has precision and sensitivity at least equivalent to that of the Jaffé reaction (for review see Spencer, 1986). The method was free of interferences from acetoacetic acid, ascorbic acid, bilirubin, and hemoglobin (Margrey et al., 1984). For serum samples, bilirubin is always present and absorbs slightly at 340 nm, the wavelength used for the NADH detection. This can be neglected by measuring the reaction rate. Creatine and pyruvate in serum influence the rate, but the preincubation of the reaction mixture can remove this effect.

2.2.2.2 Methods using creatininase and creatinase

The combined use of creatininase and creatinase became possible to measure creatinine when bacterial sarcosine oxidase (sarcosine: oxygen oxidoreductase, EC 1.5.3.1) was found to be commercially available (Suzuki, 1981). The method is based on the following principle: The degradation of creatinine and its products is sequentially catalyzed by creatininase, creatinase, and sarcosine oxidase (Kinoshita and Hiraga, 1980; Fossati et al., 1983: Suzuki and Yoshida, 1984). H_2O_2 produced can be detected by the peroxidase-coupled color development.

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatininase}} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{Creatinase}} \text{Sarcosine} + \text{Urea}$$

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{\text{Sarcosine oxidase}} \text{Glycine} + H_2O_2 + \text{HCHO}$$

$$H_2O_2 + \text{leuco dye} \xrightarrow{\text{Peroxidase}} \text{colored dye} + H_2O$$

A multicenter evaluation of this method was performed in 16 clinical laboratories in the world and the manufacturer's laboratory (Guder et al., 1986). This method permits a precise and specific determination of creatinine in serum and urine. Substantially better interlaboratory agreement was observed with this method than with the enzymatic UV test and various Jaffé procedures (Guder et al., 1986). Well-known interferences in the measurement of creatinine with Jaffé procedures, e.g. ketone bodies, cephalosporin antibiotics and noncreatinine chromogens had no effect on this methods (Suzuki and Yoshida, 1984; Guder et al., 1986; Goren et al., 1986; Roberts, 1988). Recently Fushimi et al. (1992) reported that ethamsylate has negative interference. As ethamsylate contains the hydroquinone structure, hydrogen peroxide produced will be consumed by this compound to show the interference.

Yasuhara et al. (1982) reported the method to determine creatine by using sarcosine oxidase and formaldehyde dehydrogenase:

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{\text{Sarcosine oxidase}} \text{Glycine} + H_2O_2 + HCHO$$

$$HCHO + H_2O + NAD^+ \xrightarrow{\text{Formaldehyde dehydrogenase}} HCOOH + NADH + H^+$$

This method can be used to detect creatinine by the addition of creatininase and creatinase to the assay mixture.

2.2.2.3 Methods using creatinine deiminase

Creatinine deiminase (creatinine iminohydrolase, EC 3.5.4.21) catalyzes the conversion of creatinine to N-methylhydantoin and ammonia (Szulmajster, 1958).

$$\text{Creatinine} \rightarrow \text{N-methylhydantoin} + NH_3$$

The amount of creatinine can be determined by measuring ammonia produced. The different approaches to ammonia detection were reported (Spencer, 1986). These are an ammonia gas-sensing electrode, pH electrode, Berthelot reaction (see Spencer, 1986; Bolleter et al., 1961) and glutamate dehydrogenase. Ammonia in the samples interferes the creatinine determination by this method. Ammonia content in serum is low, but that in urea must be removed before measurement. 5-Fluorocytosine which are used as antimicrobial drug was reported to have positive interference by this method (Noble et al., 1984).

Shimizu et al. (1989) proposed a novel enzyme system to detect creatinine. They purified microbial enzymes to catalyze the following sequence of change of creatinine:

$$\text{Creatinine} \xrightarrow{\text{creatinine deiminase}} \text{N-methylhydantoin}$$

$$\text{N-methylhydantoin} \xrightarrow[\text{amidohydrolase}]{\text{ATP} \rightarrow \text{ADP}} \text{N-carbamoyl sarcosine} \xrightarrow[\text{amidohydrolase}]{\text{N-carbamoylsarcosine}} \text{sarcosine}$$

The amount of sarcosine and ADP produced is directly proportional to that of creatinine in the sample. Sarcosine and ADP can be detected by the methods as described above. Inteferences from creatine and ammonia can be excluded by the method.

2.2.3 Preferred method

The choice of the method for creatinine determination must meet several criteria. Most important is accuracy, especially for specimens in which a mixture of potential interferents is likely to be present (Murray, 1987). As described in the above sections, many modifications of Jaffé procedures have suggested to eliminate inteference by many substances in serum, but with imcomplete success (Narayanan and Appleton, 1980; Bowers and Wong, 1980; Spencer, 1986). So the enzymatic methods were developed. Of the coupled-enzyme systems, the peroxidase-coupled method is superior because of its sensitivity and easy application to the automated analyzer. Comparison of the Jaffé procedures with the enzymatic method was performed by various laboratories (Guder et al., 1986; Goren, 1986; Lindback and Bergman, 1989; Watts and Pillay, 1990; Jacobs et al., 1991). Guder et al. (1986) evaluated the enzymatic method in various laboratories and compared with the Jaffé method. They showed that the enzy-

matic method had a much better interlaboratory comparability than Jaffé method. Well-known interferences in the measurement of creatinine with Jaffé procedures, such as ketone bodies, cepharosporin antibiotics, and other non-creatinine chromogens had no effect on the enzymatic method. Jacobs et al. (1991) compared the two assays for a series of animal sera and found that the enzymatic method was less suceptible to the inteferences caused with Jaffé method. The only instances where two methods were similarly affected were inteferences caused by bilirubin and, to lesser extent, lipid. Bilirubin caused a decrease in apparent creatinine concentration. Other laboratories also reported that the enzymatic method is superior to the Jaffé method. So, the enzymatic method could be used in place of the Jaffé reaction for routin work.

3. Sarcosine oxidase

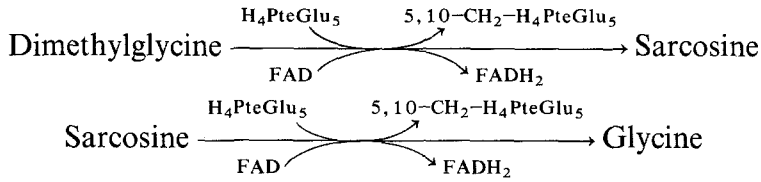
3.1 Sarcosine oxidizing enzymes

Dimethylglycine dehydrogenase (EC 1.5.99.2) and sarcosine dehydrogenase (EC 1.5.99.1) were purified from liver mitochondria (Frisell and Mackenzie, 1962; Sato et al., 1979; Wittwer and Wagner, 1980, 1981a). Sarcosine dehydrogenase was also solubilized from bacteria (Pinto and Frisell, 1975; Oka et al., 1979). Dimethylglycine dehydrogenase and sarcosine dehydrogenase from liver mitochondria have been shown to be folate-binding proteins (Wittwer and Wagner, 1980, 1981a,b) and cotain covalently bound FAD (Table 1). These enzymes have

Table 1. Sarcosine oxidizing enzymes

	Mr	Prosthetic group	Km (mM)	Ref
Sarcosine Dehydrogenase				
liver mitochondria	99,000 (monomer)	covalent FAD	0.5	Sato et al., 1979 Cook, Wagner, 1986
Pseudomonas pudida	170,000 (homo-tetramer)	flavin	29	Oka et al., 1979
Pseudomonas sp. WRF		covalent FAD		Pinto, Frisell, 1975
Sarcosine Oxidase				
Cylindrocarpon didum M-1	45,000 (monomer)	covalent FAD	1.8	Mori et al., 1980
Bacillus sp. B-0618	42,000 (monomer)	covalent flavin	12.2	Matsuda et al., 1987
Bacillus sp. NS-129	42,955 (387 amino acid residues)	covalent FAD		Koyama et al., 1991
Streptomyces sp. KB210-8SY	44,000 (monomer)		0.91	Inouye et al., 1987
Alcaligenes denitrifcans	190,000 (hetero-dimer)	flavin	4.2	Kim et al., 1987
Corynebacterium	174,000 (hetero-tetramer)	covalent FAD noncovalent FAD	3.4	Suzuki, 1981
Arthrobacter denitrificans	185,000 (hetero-tetramer)	covalent flavin noncovalent flavin	6.4	Ogushi et al., 1988

been known to be related to the choline metabolism (Wittwer and Wagner, 1981b). The last two steps of choline degradation are oxidative demethylation reaction catalyzed by dimethylglycine dehydrogenase and sarcosine dehydrogenase. It is proposed that a methylene group is generated in both of these reactions as a result of the oxidation of the N-methyl group.



where $H_4PteGlu_5$ and 5,10-CH_2-$H_4PteGlu_5$ represent tetrahydropteroylpentaglutamte and 5,10-methylenetetrahydropteroylpentaglutamte, respectively. In vivo, the enzyme presumably use the bound $H_4PteGlu_5$ to accept the methylene groups with the formation of 5,10-CH_2-$H_4PteGlu_5$.

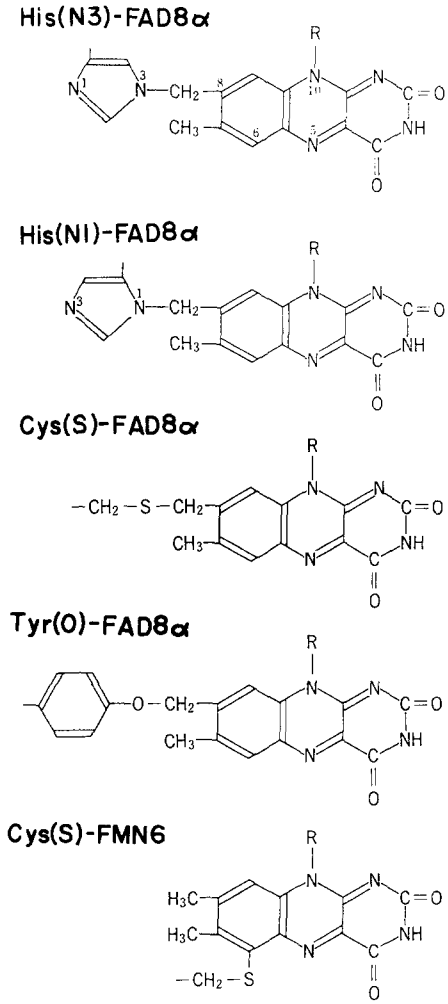


Fig. 3. The flavin-amino acid bonds found in flavoproteins (Decker, 1982)

Primary structure around covalent FAD of sarcosine oxidizing enzymes

SD P. sp. WRF[1)]	-Asp-Lys-Ser-Glu-Gly-His*-(Asp, Ala, Thr)
SD rat liver[2)]	-Leu-Thr-Ser-Gly-Thr-Thr-Trp-His*-Thr-Ala-Gly-Leu-Gly-Arg
SO[3)]	-Ala-Gly-Ile-Ala-Cys-Lys-Asp-His*-Val-Ala-Thr-Ala-Phe-Ala

[1)] Sarcosine dehydrogenase from *Pseudomonas* sp. WRF (Pinto and Frisel, 1975)
[2)] Sarcosine dehydrogenase from rat liver mitochondria (Cook et al., 1985)
[3)] Sarcosine oxidase from *Corynebacterium* sp. U-96 (Suzuki and Kawamura-Konishi, 1991)

Fig. 4. Sequence of FAD-binding site of sarcosine oxidizing-enzymes

Sarcosine oxidase catalyzes the oxidative demethylation of sarcosine using molecular oxygen as an electron acceptor, Sarcosine oxidase must have a role similar to that of sarcosine dehydrogenase, since *Corynebacterium* enzyme has been shown to be a folate-binding protein (Kvalnes-Krick and Jorns, 1987). Involvement of sarcosine oxidase in the metabolism of creatinine (Kim et al., 1986; Shimizu et al., 1989) and choline (Mori et al., 1980) in microorganisms was clearly shown. Especially Kim et al. (1986) reported a novel metabolic pathway for creatinine degradation, with N-methylhydantoin, N-carbamoylsarcosine, sarcosine, and glycine as the successive degradation metabolites. Futhermore, they purified all the enzymes involved in the degradation and proposed an enzymatic method for creatinine assay as described above (Shimizu et al., 1989).

Sarcosine oxidizing enzymes so far studied contain covalently bound flavin. The bound flavin and the protein moiety have been shown to be linked via the 8 α-position of the isoalloxazine ring to the imidazole N (3) of a histidine residues (Fig. 3; Cook et al., 1980; Pinto and Frisell, 1975; Hayashi et al., 1982; Decker, 1982). The amino acid sequences around the covalently bound FAD of sarcosine dehydrogenase from liver mitochondria (Cook et al., 1985) and *Pseudomonus* (Pinto and Frisell, 1975), and of *Corynebacterium* sarcosine oxidase (Suzuki and Kawamura-Konishi, 1991) were compared. As Fig. 4 shows, there is no sequence homology among them.

As Table 1 shows, the sarcosine oxidases can be classified into three groups, on their subunit composition, that is, monomer, heterodimer, and heterotetramer. They all contain a polypeptide of approximate Mr 50,000, but their primary sequences seem to be different among different groups, since no homologous sequence was observed between the partial sequence of subunit B of *Corynebacterium* enzyme and the complete primary strucuture of *Bacillus* enzyme. As described in the following sections, it becomes clear that the functional domain of the *Corynebacterium* enzyme is in subunit B (Mr 45,000 subunit). It remains to be open for further study on the role of the other subunits in the *Corynebacterium* enzyme, especially the largest subunit (Mr 100,000).

3.2 Primary structure of sarcosine oxidases

Koyama et al. (1991) cloned the sarcosine oxidase gene from *Bacillus* sp. NS-129 and expressed the gene in *Escherichia coli*. They determined the nucleotide sequence of the gene and deduced the amino acid sequence. It was shown that

the enzyme is composed of 387 amino acid residues. This is the only enzyme that the complete sequence of sarcosine oxidase has been published in the journal so far. The enzyme contains the covalently bound FAD, but the amino acid residue at the binding site is not yet determined.

3.3 Reaction mechanism of Corynebacterium sarcosine oxidase

3.3.1 Overall reaction

Among sarcosine oxidases studied so far, *Corynebacterium* enzyme has been studied extensively. Hayashi et al. (1983a) proposed an overall reaction mechanism by measuring the oxygen uptake at various concentration of oxygen and sarcosine. The enzymatic reaction follows the ping-pong bi-bi mechanism.

$$E_o + S \rightleftharpoons E_oS \rightleftharpoons E_rP \rightarrow E_r + P$$

$$P + H_2O \xrightarrow{\text{spontaneously}} \text{Glycine} + HCHO$$

$$E_r + O_2 \rightleftharpoons E_rO_2 \rightleftharpoons E_oH_2O_2 \rightarrow E_o + H_2O_2$$

where E_o and E_r represent the oxidized and reduced forms of the enzyme, respectively, and S and P represent substrate sarcosine and its possible intermediary reaction product, $CH_2{=}N{-}CH_2COOH$, respectively.

3.3.2 Oxidation and reduction of the bound FADs

Corynebacterium enzyme is unique in that 1) it contains one covalently bound and one noncovalently bound FADs and 2) consists of 4 non-identical subunits. Noncovalent FAD can be removed by acid-ammonium sulfate treatment of the enzyme. The enzyme was named "semiapoenzyme" (Hayashi et al., 1980). Changing the time of treatment of the enzyme with acid-ammonium sulfate, we can get a semiapoenzyme that contains various amounts of noncovalent FAD. From the analyses of the reaction of dithiothreitol with FADs of the semiapoenzyme and the fact that the semiapoenzyme had practically no activity for sarcosine oxidation (Hayashi et al., 1980, 1985), the different role of two types of FAD in the enzyme was proposed. The noncovalent FAD accepts electrons directly from dithiothreitol (sarcosine) while the covalent FAD accepts electrons only from the reduced noncovalent FAD. The different role of two types of flavins were also shown by Jorns (1985). Fifty percent of the enzyme flavin forms a reversible covalent complex with sulfite and the enzyme loses completely its activity. The sulfite did not prevent the reduction of the sulfite-unreactive flavin by sarcosine but interfered the reoxidation of reduced enzyme by oxygen. From these observations, it was proposed that noncovalent FAD reacts with sarcosine, and covalent FAD with molecular oxygen (Jorns, 1985). That is, electrons flow from sarcosine to oxygen as:

Sarcosine → noncovalent FAD → covalent FAD → oxygen

Kawamura-Konishi and Suzuki (1987) measured the rate of reduction of the bound FADs of the enzyme with the stopped-flow method. The rate was determined by measuring the absorbance change at 455 nm under anaerobic condi-

tions. The time courses consisted of three first-order reactions. The slowest rate was too small to explain the rate of overall reaction, so the rate was not considered to explain the overall rate. The maximum rate of reduction at the infinite sarcosine concentration were 31 and 6.7 s^{-1}. The rate of oxidation of the reduced flavin moieties was 100 s^{-1}. To explain the rate of overall reaction (18 s^{-1}), the following mechanism was proposed.

(Reduction)

$$E_o^o + S \rightleftharpoons E_o^o S \rightarrow E_o^r + P \rightarrow E_r^0 + P \quad (1)$$

$$E_r^0 + S \rightleftharpoons E_r^0 S \rightarrow E_r^r + P \quad (2)$$

(Oxidation)

$$E_r^r + O_2 \rightleftharpoons E_r^r O_2 \rightarrow E_o^r + H_2O_2 \rightarrow E_r^0 + H_2O_2 \quad (3)$$

$$E_r^0 + O_2 \rightarrow E_o^o + H_2O_2 \quad (4)$$

Where E represnts sarcosine oxidase, its superscript and subscript represent the noncovalent and covalent FADs, respectively, and the term "r" and "o" represent the reduced and oxidized forms of the bound FAD, respectively, P is the reaction product, presumably $CH_2{=}N{-}CH_2COOH$. The half-reduced form of the enzyme, E_r^0, is distributed between two pathways. One is that E_r^0 reacts with sarcosine to change the fully reduced form E_r^r, which reacts with molecular oxygen to regenerate the oxidized form of the enzyme (reactions 1, 2, 3, and 4). The other is that E_r^0 reacts with molecular oxygen to regenerate the fully oxidized form of enzyme. The overall rate was explained by assuming that 70% of the enzyme acts via the fully reduced form, and 30% of the enzyme did so without forming the fully reduced enzyme (Reactions 1 and 4).

Ali et al. (1991) reported the oxidation-reduction rates of the bound flavins similar to Kawamura-Konishi and Suzuki (1987). But they observed the semiquinoid form during the reduction and proposed that the step of the formation of the semiquinoid form is limiting the rate of overall reaction. These two

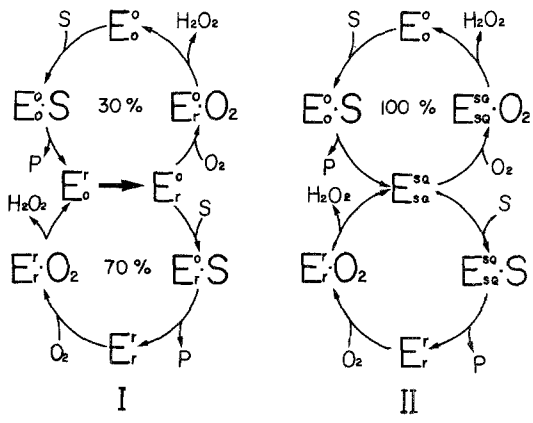


Fig. 5. Reaction mechanism of sarcosine oxidase according to the reports of Kawamura-Konishi and Suzuki (1987) (Scheme **I**) and Ali et al. (1991) (Scheme **II**). Details are in the text

proposals were compared schematically in Fig. 5 according to their mechanisms presented. The difference in the results between two groups must be due to the difference in the preparation of the enzyme used, since the steady state kinetics was different between two groups, that is, Hayashi et al. (1983a) observed a series of parallel lines of Lineweaver-Burk plot at various concentrations of oxygen, but Jorns' did not (Ali et al., 1991). Moreover, if the enzyme turnovers by the cyclic change between the fully oxidized and the semiquinoid form, the difference spectrum of enzyme (the steady state level minus the reduced level) detected by the stopped-flow method must be that of the oxidized minus semiquinoid forms. Though Ali et al. (1991) did not report this type of experiment, Kawamura-Konishi and Suzuki observed the difference spectrum between the oxidized and the fully reduced forms of enzyme by the stopped-flow method, supporting their proposed mechanism (Scheme I in Fig. 5).

3.3.3 Active site structure of *Corynebacterium* sarcosine oxidase

Corynebacterium enzyme was inhibited by sulfhydryl reagents and a competitive inhibitor, acetate prevented the inhibition (Hayashi et al., 1983b). Suzuki and Kawamura-Konishi (1987) labeled the enzyme with [^{14}C] iodoacetamide (IAM) and found that [^{14}C] IAM was specifically incorporated into the B subunit of the enzyme. Almost two mole of [^{14}C] IAM was incorporated into one mole of the enzyme, and acetate reduced the incorporation to nearly 50%. To see the structure around the labeled Cys residues of the enzyme, Suzuki and Kawamura-Konishi (1991) labeled the enzyme with [^{14}C] IAM in the presence and absence of sodium acetate. The ^{14}C-labeled subunit B was extensively digested with trypsin and chymotrypsin and the digests were analyzed by HPLC. As Fig. 6

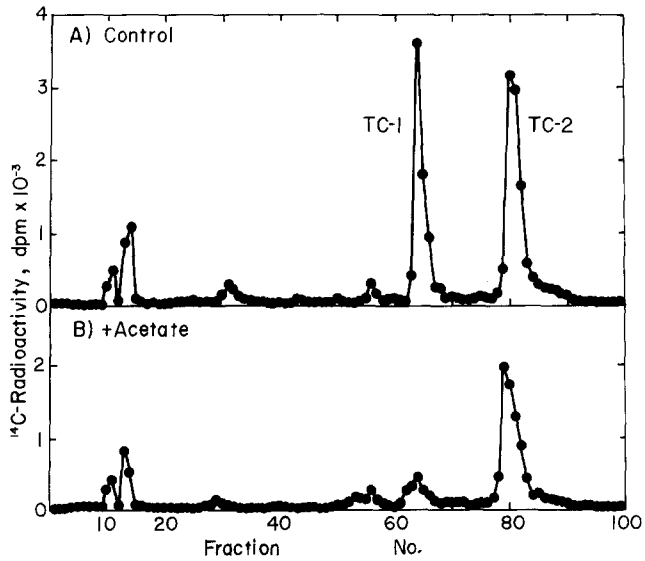


Fig. 6. HPLC profiles of the tryptic and chymotryptic digests of [^{14}C]-labeled subunit B. The labeled subunit was obained by incubating the enzyme with [^{14}C] IAM in the presence (**B**) and absence (**A**) of sodium acetate

shows, two labeled peaks were observed in the absence of sodium acetate, but the labeling in TC-1 peak was negligible in the presence of sodium acetate. The amino acid sequences of these peptides were;

TC-1: Cys-Gly-Thr-Pro-Gly-Ala-Gly-Tyr

TC-2: Ala-Gly-Ile-Ala-Cys-Lys-Asp-His-Val-Ala-Thr-Ala-Phe

The sequence of TC-2 contains the sequence of the covalent FAD-binding site reported by Shiga et al. (1983). Therefore one of the Cys residues labeled by [^{14}C] IAM locates near the covalent FAD-binding site. To know the amino acid sequence around these two peptides in much larger fragments, ^{14}C-labeled subunit B was treated with CNBr and the treated subunit B was analyzed by HPLC. ^{14}C-labeled peptides were purified and the N-terminal amino acid sequences were analyzed. One peptide containing TC-2 sequence was fluorescent, confirming that the sequence contains the covalent FAD-binding site. The other peptide contained TC-1 sequence and had a common sequence of AMP-binding site of other flavoproteins. Combining the result with the fact that IAM-labeling was inhibited by sodium acetate, a competitive inhibitor for sarcosine, Suzuki and Kawamura-Konishi (1991) proposed that the noncovalent FAD locates at the sarcosine-binding site near the TC-1 sequence.

Suzuki and Kawamura-Konishi (1991) studied the kinetic properties of these two types of the enzyme which were obtained by treating the enzyme with IAM in the presence and absence of sodium acetate. The former refers to the IAM (+A)-enzyme and the latter to the IAM (−A)-enzyme. Reduction of FAD moiety of IAM (+A)-enzyme obeyed the rapid and very slow phases, and the extent of the fast phase was 50% of the total change and almost the same as that of the native enzyme in presence of sodium sulfite. Sodium sulfite is known to react specifically with the covalently bound FAD of the enzyme (Jorns, 1985). Reduction of the FAD moieties of the IAM (−A)-enzyme also obeyed rapid and slow phases, but the magnitude of the fast phase was very small. The enzyme activity of the IAM (−)- and IAM (+)-enzymes was explained well by assuming that 1) the concentration of active enzyme is proportional to the fractions of the rapidly reducible FAD moiety of the enzyme and 2) by IAM treatment of enzyme mainly noncovalent FAD becomes to function in catalyzing the oxidation of sarcosine (Suzuki and Kawamura-Konishi, 1991).

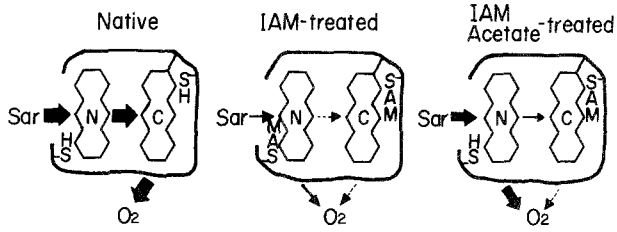


Fig. 7. Schematic presentation of electron transport pathway of control and iodeacetamide-treated enzymes. IAM-Acetate-treated and IAM treated-enzymes refer to the enzyme treated with IAM in the presence and absence of sodium acetate, respectively

4. Conclusion

1) The Jaffé procedure for the determination of creatinine has long been used in the clinical laboratories. But the method suffers from various interferences, such as ketones, ketonic acids and cephalosporin antibiotics. Enzymatic methods were developed to overcome the problem and the peroxidase-coupled procedure has a practical advantage in comparison with other enzymatic methods because of its sensitivity and easy application to the automated analyzer. Therefore, it is recommended to replace the chemical method to the enzymatic method.
2) Sarcosine oxidase has a critical role in the enzymatic method. The enzyme from *Corynebacterium* contains noncovalently and covalently bound FADs, and the noncovalent FAD functions as a dehydrogenase flavin and the covalent as an oxidase one. Modification of enzyme with iodoacetamide changed the enzyme so that mainly noncovalent FAD functions in the oxidation of sarcosine. Moreover, it now appeared that two SH groups locate at the active site of the enzyme, one at a sarcosine-binding site (noncovalent FAD) and the other at an oxygen-binding site (covalent FAD). It is clear that further works are required for the sarcosine oxidases especially on the structural and functional aspects.

Note added in proof

Suzuki et al (1992) and Nishiya and Imanaka (1993) reported on the cloning and sequencing of monomer-type sarcosine oxidases. The homology of sarcosine oxidases between *Arthrobacter* sp. TE1826 (Nishiya and Imanaka, 1993) and *Bacillus* sp. NS-129 (Koyama et al. 1991) was 82%, but that of Arthrobacter (Nishiya and Imanaka, 1993) and *Streptomyces* sp. KB210-8SP (Suzuki et al., 1992) was 36%. Moreover, Chulmsky et al. (1993) cloned the genes encoding the four subunits of *Corynebacterium* sarcosine oxidase and sequenced the gene for the B subunit. The deduced primary structure of the B subunit agreed well with the partial amino acid sequence by Suzuki and Kawamura-Konishi (1991).

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