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THE INTERACTION OF AMINOGROUPS WITH PYRROLOQUINOLINE QUINONE AS DETECTED BY THE REDUCTION OF NITROBLUE TETRAZOLIUM

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SUMMARY The interaction of pyrroloquinoline quinone (PQQ) with amino groups was followed by measuring the capacity of adducts to reduce nitroblue tetrazolium (NBT). Of the natural amino acids only glycine, ornithine, and lysine interacted strongly with PQQ. The reducing acivity of other less reactive amino acids, but not of lysine, was increased by ammonia, primary or secondary amines. Divalent cations, in contrast inhibited development of NBT-reducing activity. PQQ also developed NBT-reactivity in the presence of seotonin and albumin. A reaction scheme is proposed which explains these findings. It is suggested that the NBT-reducing activity of plasma which is not caused by glycation of plasma proteins, arises from PQQ adducts inherrent to plasma. This NBT-reducing activity corresponds to approximately 10 ug PQQ/ml plasma.

INTRODUCTION Pyrroloquinoline quinone (PQQ, also called methoxatin) is the coenzyme of a variety of copper containing oxidoreductases (1). It was originally discovered in bacterial enzymes (2) and later identified in enzymes of higher organims such as bovine plasma amine oxidase (3), the lysyl oxidases of bovine aorta (4) and human placenta (5), or in the porcine kidney diamine oxidase (6). It is not known whether PQQ is synthesized in higher organisms or whether a vital requirement exists for this cofactor.

In the latter case PQQ should be present in blood. Because PQQ readily forms adducts with nucleophilic compounds (7), PQQ is not likely to be present in blood in its free form. We report that PQQ adducts reduce NBT.

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Abbreviations: PQQ, pyrroloquinoline quinone; NBT nitroblue tetrazolium; PBS, phosphate buffered saline; EDTA, ethylenediamine tetraacetic acid.

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Using this property we studied the interaction of PQQ with amino acids and proteins.

METHODS

Materials Plasma was obtained from blood collected in EDTA. Methoxatin (PQQ) was purchased from Fluka (Buchs, Switzerland); human albumin, 'reinst' from Behringwerke, (Marburg, FRG); the animal albumins, egg albumin and the amino acids from Sigma; the methyl ester of glycine and alanine as well as glycinamide from Bachem (Bubendorf, Switzerland). The NBT reagent was provided by Fa. Hoffmann LaRoche. All other chemicals were of commercial origin and of the highest purity available.

Determination of reducing activity One hundred ul of the amino compound (0.2 mol/l) were combined with 100 ul PBS with or without an inhibiting or activating substance and 100 ul of PQQ (2.76 mmol/l in PBS). Unless otherwise stated, 100 ul of this mixture were added immediately to 1 ml NBT reagent (0.25 mol/l NBT in 0.1 mol/l carbonate buffer, pH 10.35). Reduction of NBT at 37°C was followed spectrophotometrically at 530 nm for 15 minutes. Reducing activity was calculated as the absorbance change between 10 and 15 minutes.

RESULTS

In phosphate buffered saline PQQ did not reduce NBT. Addition of amino acids caused NBT-reactivity to arise. The potency of various amino compounds in inducing this NBT-reducing activity was investigated at a 70 molar excess of amino compound over PQQ. Serotonin was most reactive, followed by glycine, ornithine, lysine, and citrulline (Figure 1; Table 1).

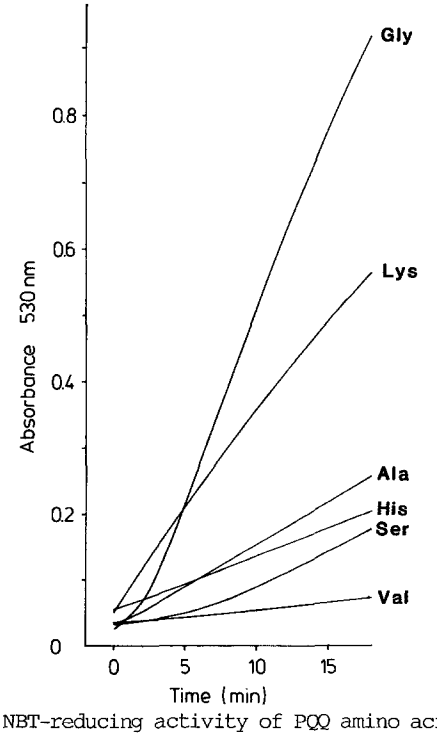


Figure 1: NBT-reducing activity of PQQ amino acid mixtures.

Table 1: Effect of Compounds which increase or decrease NBT-reactivity caused by PQQ amino-compound interaction

Substance	Concentration	Reducing activity (dA 530nm)
Glycine	70 mM	0.26
+ $MgCl_2$	70 mM	0.08
Serine	70 mM	0.05
+ NH_4Cl	70 mM	0.07
"	166 mM	0.09
"	333 mM	0.17
+ iminodiacetic acid	70 mM	0.07
Lysine	70 mM	0.17
+ NH_4Cl	70 mM	0.17
5-OH-Lysine	70 mM	0.12
Ornithine	70 mM	0.30
Citrulline	70 mM	0.05
beta-Alanine	70 mM	0.00
Serotonin, oxalate	70 mM	0.64
", creatinine sulfate	70 mM	0.26
Albumin, human	13 mg/ml	0.07
+ NH_4Cl	70 mM	0.07
+ $MgCl_2$	70 mM	0.04

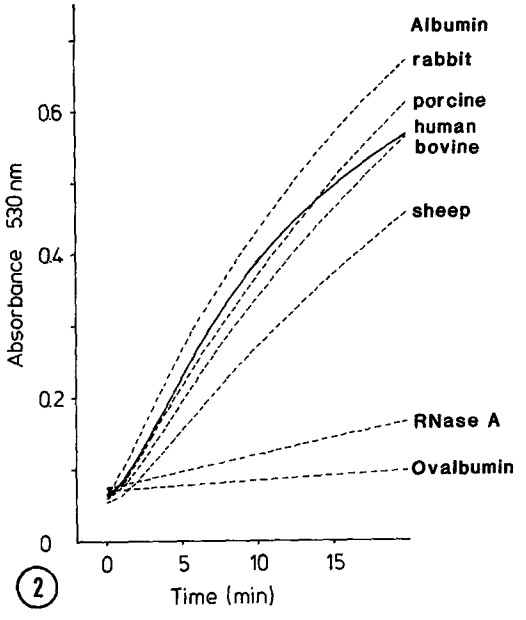
The PQQ adduct formed with glycine was yellow and those with serotonin or the ε-amino group containing amino acids were of brownish color. When kept overnight and then assayed a precipitate formed in the NBT assay.

Proline, glycinamide and the methylester of alanine did not produce NBT-reactivity. In contrast, the glycinemethylester was reactive, probably because of ester hydrolysis. The NBT-reducing activity of the less reactive amino acids was increased by ammonia or iminodiacetic acid while divalent cations inhibited it (Table 1).

When PQQ was added to albumin, NBT-reactivity immediately appeared (Figure 2). It did not increase if interaction was allowed to proceed for 1 hour before NBT-assay but decreased with longer incubation times. It was 84% of the initial value after 1 day and after two days 66%.

PQQ did not develop similar NBT-reducing activity with ovalbumin or ribonuclease. The NBT-assay was also performed in the presence of ovalbumin in order to prevent formation and/or precipitation of the diformazan. The reducing activity of PQQ glycine or lysine mixtures was not affected by the presence of ovalbumin.

Addition of PQQ to plasma caused NBT-reducing activity to increase in proportion to the amount of PQQ added. Addition of 20 ug PQQ/ml plasma caused



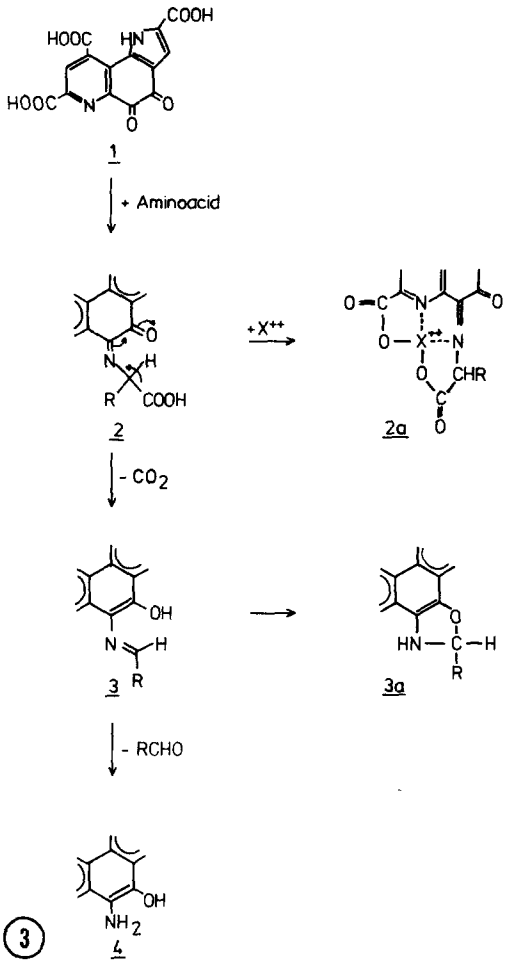


Figure 2: NBT-reducing activity of PQQ in the presence of proteins.

Figure 3: Reaction scheme for the interaction of PQQ with amino groups: 1 PQQ; 2 ketoimine adduct; 3+4 NBT-reducing eneaminols 3 aldimine; 4 amino-PQQ. 2a complex with divalent cation X^{++} 2b oxazole.

an additional absorbance change corresponding to that observed with plasma of control persons (data not shown).

DISCUSSION

Ketoamines are potent reducing agents in alkaline medium (8). Under alkaline conditions the reactive eneaminol is formed from the ketoamine which, with NBT, affords reductive imidazol ring opening. NBT is converted to the monoformazan and to some extent also to the water insoluble diformazan. Because glycated proteins contain the ketoamine (also called fructosamine) the NBT-reaction is being used for measurement of glycation of plasma proteins (9). The reducing eneaminol is also present in amino-PQQ and we

therefore used the NBT-assay for detection of the interaction between PQQ and compounds with reactive amino groups.

Formation of the ketoimine 2 occurs readily with the α-amino group of amino acids because of the comparably low pK-value. This ketoimine does not posses reductive properties while the aldimine 3 does (Figure 3).

The ketoimine 2 combines the metal ion chelating structures of the Schiff's base and that of 2,6-pyridinedicarboxylic acid. It seems therefore reasonable to explain the inhibitory effect of divalent cations by prevention of double bond migration and aldimine 3 formation because of complex 2a formation.

The aldimine 3 may either react to amino-PQQ with liberation of a carbonyl compound or get converted to the oxazole 3a (1; J.A. Duin, personal communication). These nonreducing oxazoles apparently produce the precipitate observed if the NBT-reaction is performed on reaction mixtures kept at room temperature overnight.

It seems unlikely that significant amounts of amino-PQQ 4 are formed under the conditions used in our study. Prolonging the time for reaction did not increase NBT-reducing activity. This is in contrast to the situation with cationic micells where carbonyl compounds are formed during 24 h at 30°C (10). Whether liberation of PQQ may arise under the alkaline conditions of the NBT reaction and the redox cycle may continue remains to be established. Preliminary work with pig kidney diamine oxidase in the presence of alkaline NBT and glycine indicates that it does.

The finding that the reactivity of the less reactive amino acids, but not of lysine is enhanced by ammonia shows that the high reactivity of lysine is caused by its ε-amino group through the mechanism by which ammonia affords activation. The remarkable reactivity of serotonin with PQQ indicates that the secondary ring nitrogen activates PQQ to an extent that condensation with the primary amino group becomes possible. It thus appears that the reactivity of diamines and indolamines with PQQ is a consequence of cofactor activation by a specific structural feature of its substrate. This may help to explain why aminooxidases act on primary amino groups in spite the unfavourably high pK value.

Of the proteins investigated only albumin was found to interact significantly with PQQ. RNase which posses a free low-pK amino terminus is only marginally reactive and ovalbumin, in which the amino end is acetylated, shows almost no NBT-reactivity. Albumin therefore posses a unique amino group for interaction with PQQ. Such reactivity could arise at an amino group close to an activator group. With the activator at the proper place activation by ammonia would not occur.

How exactly this activation by ammonia and other protonated amino groups works is not clear. It has been speculated that ammonia could interact with the carbonyl group at C-9 and bring about out-of-plane rotation of this carboxylic acid group creating a p-quinone structure (1).

The NBT-reducing activity generated by addition of PQQ to plasma allows estimation of maximal inherrent plasma PQQ levels. About half of the NBT-reducing activity of plasma without added PQQ is explained by the presence of glycated plasma proteins containing the ketoamine (fructosamine) structure which is able to directly reduce NBT. The remaining activity is caused by unknown, non-dialyzable components which seem not to be associated with albumin and differ from person to person (11).

If it is assumed that this residual NBT-activity is entirely caused by PQQ-adducts or quinoproteins, comparison of the unaccounted absorbance with that obtained by addition of PQQ to plasma yields an estimate of about 10 ug of PQQ/ml of plasma. Because the structure and stability of biological PQQ adducts is not yet known, direct measurement of PQQ in biological fluids has not been possible. We are developing methods for the direct demonstration of PQQ-containing adducts. This will help to clarify the issue whether PQQ is simply a new coenzyme or whether it also represents a new vitamin for man and other animals.

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